ROOM TEMPERATURE PHOSPHORESCENCE, FUNDAMENTAL ASPECTS AND APPLICATIONS

BY

MARTA MARÍA ANDINO PADILLA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
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Applications and fundamental aspects of room temperature phosphorescence (RTP) are discussed. Room temperature phosphorescence is applied to the determination of compounds of biological importance: several indoles derivatives, including some indolecarboxylic acids, and diazepam. The optimum experimental conditions for the RTP analysis of the compounds are determined and the figures of merit evaluated. The absolute limits of detection ranged between 0.2 and 4.2 ng for the analysis of the indoles and between 0.5 and 1.9 ng for diazepam, depending on the experimental conditions. The results demonstrate that RTP constitute a simple and sensitive analytical method for the determination of these compounds. Room temperature phosphorescence is evaluated as a rapid and selective screening method for toxic levels of diazepam in serum and for the analysis of diazepam in tablet formulation.

The substrate, pH, and heavy atom effects on the RTP of the indoles, diazepam, caffeine and theophylline are observed. The RTP excitation and emission wavelengths do not change significantly but the intensities of the signals are greatly affected by the microenvironment that surrounds the phosphor. The heavy atom effect is necessary to obtain analytically useful RTP signals from the compounds studied. Several ion-exchange and pure cellulose filter papers are evaluated as substrate. The importance of the chemical reactions occurring at the wet surface, at the moment of spotting, and the probable existence of several hydrogen bonding and heavy atom-support interactions are discussed.

X-ray photoelectron spectroscopy studies of Whatman No. 1 filter paper are performed before and after the spotting of a luminescent compound and/or heavy atom solutions on the surface of the paper. Variations in the binding energies and the elemental ratios provide information concerning changes in the surface composition. The extent of penetration of the lumiphor and heavy atom onto the bulk of the paper is discussed. A considerable improvement in the retention of the heavy atom on the surface is obtained through the use of surfactant salts of the heavy atoms.

CHAPTER 1
THEORETICAL AND PRACTICAL ASPECTS OF SOLID SUBSTRATE ROOM TEMPERATURE PHOSPHORIMETRY

Introduction

Although solid substrate room temperature phosphorimetry has been found to be a very simple and sensitive method of analysis for compounds such as drugs, pesticides, polyaromatic hydrocarbons (PAHs), and other compounds of biological and environmental concern, its application as a routine method of analysis has been very limited. The absence of a theoretical model and the problems associated with the commonly used substrates (background phosphorescence and matrix-related problems which affect the precision and accuracy) has probably limited its acceptance by the scientific community. In order to develop an analytical model, an understanding of the surface processes that make possible the observation of room temperature phosphorescence is needed. Many studies on the RTP behavior of different compounds on different substrates have been reported and various theories on the types of interactions present at the surface (e.g., hydrogen bonding and matrix isolation) have been postulated. We now have a better understanding of the mechanisms of interactions at the surface, but additional studies are needed in this area if basic questions concerning the different theories are to be answered. A knowledge of the factors that influence the phosphorescence properties of luminescent compounds will help to elucidate the chemical interactions

occurring at the surface and answer some of the basic questions concerning the RTP process. This will eventually result in the development of an analytical model suitable for the application of RTP to "real life samples" on a routine basis.

In this dissertation both fundamental aspects and analytical applications of RTP on solid supports will be covered.

Phosphorescence and the Triplet State

Population of the Triplet State

Phosphorescence is a luminescence process. It involves the radiative emission from a molecule undergoing a transition from an upper electronic state to a lower electronic state of different multiplicity. In organic molecules phosphorescence usually occurs from molecules with a rigid molecular skeleton, a highly conjugated system, and large energy separation between the lowest excited triplet state and the ground state (1). Since the ground state of most organic molecules is a singlet state, direct excitation from the ground state into the triplet state is forbidden by the spin selection rule which does not allow transition between electronic levels of different multiplicities (1,2). The triplet state is achieved almost exclusively via excitation into a singlet state by absorption of UV-Vis radiation.

The main photophysical processes involved in the population and deactivation of the triplet state are absorption (A), vibrational relaxation (VR), internal conversion (IC), fluorescence (F), intersystem crossing (ISC), and phosphorescence (P). These are illustrated in Fig. 1.

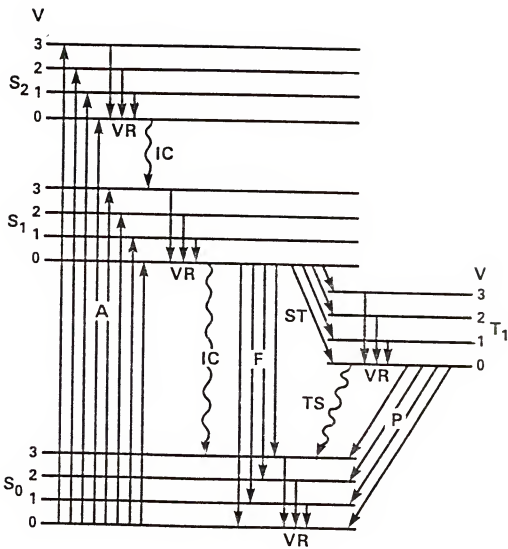


Fig. 1. Jablonskii diagram showing the different radiative and nonradiative transitions in a molecule undergoing absorption (A) from the ground electronic state (S_0) to any of the various vibrational levels (V) of the electronically excited states (S_1 and S_2). (VR, vibrational relaxation; IC, internal conversion; F, fluorescence; ST, intersystem crossing to the lowest triplet state (T_1); TS, intersystem crossing from T_1 to S_0; P, phosphorescence)

When a molecule absorbs excitation energy, it is elevated to some vibrational level of one of the excited singlet states, S_n. The excited singlet molecule relaxes rapidly via vibrational relaxation and internal conversion processes into the lowest vibrational level of the first excited state (S_1). The VR process is very fast ($< 10^{-13}$ sec); excess vibrational energy is lost as the molecule deactivates to the lowest vibrational level of the corresponding excited state (1). The IC processes occur between states of the same multiplicity and as a result of direct coupling of the lowest vibrational level of a higher excited state and the highest vibrational level of the lower excited state (see Fig. 1); IC processes are of the order of 10^{-12} sec (1). By a succession of IC processes immediately followed by VR processes, the molecule deactivates rapidly to S_1, the first excited state.

The molecule in the first excited state has a longer lifetime than higher excited states, of the order of 10^{-7} to 10^{-9} sec; it will relax into the ground state by one of several available mechanisms (1,2). The deactivation process depends on the molecular structure and on the environment that surrounds it. Internal conversion may take the molecule into a higher vibrational level of the ground state from which it relaxes into the Boltzmann vibronic distribution. Alternately, if the energy separation of the upper and lower electronic states is relatively large and the molecule does not possess many vibrational degrees of freedom, a radiative transition may occur. The emission of a photon of light as a result of transitions between electronic levels that have the same multiplicity

is called fluorescence. Fluorescence is the sister technique of phosphorescence.

Internal conversion and fluorescence are not the only processes which compete for the deactivation of the excited singlet molecule. Intersystem crossing (ISC), photochemical reactions and quenching processes may also occur. The quenching of radiation by certain extraneous species present in the environment (e.g., oxygen and nitric oxide), or the possibility of a chemical reaction of the excited singlet molecule, promotes nonradiative transitions into the ground state of the molecule. The ISC takes the molecule into the triplet excited state from which phosphorescence can be observed.

The ISC involves a change in spin multiplicity and occurs with much less probability [10^{-3}-10^{-6} that of allowed transitions (1,2)]. The molecule in an excited triplet state relaxes via VR and IC into the lowest vibrational level of the lowest triplet state (T_1). From T_1, the molecule may return to the ground state via nonradiative processes (ISC, quenching, photochemical reaction), or it may emit light (phosphorescence).

The population of the triplet state then depends on the rate of the ISC process. Since this is a transition between states of different multiplicity, it is strictly forbidden by quantum mechanics due to the selection rule requiring conservation of spin angular momentum (2,3). However, spin-forbidden transitions can occur under certain conditions. It is never really possible to have pure spin states because the spinning electron has a magnetic moment which can interact with the magnetic field associated with orbital angular

momentum. The coupling of the electron spin with the orbital angular momentum produces a mixing of states of different multiplicities. This mechanism, known as spin-orbit coupling, removes the spin-forbidden nature of the transitions. The mixing of singlet and triplet states is proportional to the spin-orbit interaction energy and inversely proportional to the energy difference between the states being mixed (3). Both factors are determined by the molecular structure and the environment that surrounds the luminescence compound (1,2,4).

Lifetimes and Quantum Yields of Phosphorescence

The molecule in the lowest excited triplet state can return to the ground state via radiative or nonradiative transitions, both of which involve a change in the spin multiplicity. Because the probability for these transitions is low, the triplet state is long-lived, lasting from 10^{-5} sec to several seconds (1). During this time, the nonradiative processes compete favorably with the radiative transitions.

If $[T_1]_0$ is the concentration of excited triplet species under steady state conditions of excitation, then $[T_1]$, the concentration at time t after the exciting light is turned off, can be expressed as (1):

$$[T_1] = [T_1]_0 \exp(-K_p t)$$

where K_p is the overall rate constant which takes into consideration all radiative and nonradiative processes.

$$K_p = (k_p + \sum_i k_{i,p} + \sum_Q k_{Q,p}\,[Q])$$

where k_p is the first order radiative constant of phosphorescence, $\sum_i k_{i,p}$ represents the sum for all nonradiative unimolecular processes which compete with phosphorescence (e.g., IC and delayed fluorescence), $\sum_Q k_{Q,p}\,[Q]$ is the sum of all the pseudo-first-order rate constants for deactivation of T_1 by molecular interaction with quenchers (e.g., oxygen), and [Q] is the concentration of the quencher.

The mean lifetime of the lowest excited triplet state, τ_p, can be defined as the time t required for $[T_1]$ to fall to 1/e of its original value. Consequently,

$$\tau_p = 1/K_p$$

The equation shows that in general the greater the number of processes contributing to the deactivation of the lowest excited triplet state and the greater the probability for these processes, the shorter the mean lifetimes of the triplet state.

Chemical and photophysical processes compete with phosphorescence. At room temperature, collisional deactivation, quenching by magnetic species (e.g., oxygen), delayed fluorescence, and photochemical reactions as well as internal conversions reduce the quantum yield (Y_p) or quantum efficiency of the phosphorescence. Y_p is defined as the number of phosphorescence photons emitted per number of photons absorbed.

The quantum yield can be expressed (4) in terms of the rate constants for the phosphorescence process and the other competing processes:

$$Y_P = Y_T [k_p/(k_p + \sum_i k_{i,p} + \sum_Q k_Q [Q])]$$

where Y_T is the quantum efficiency for the formation of the triplet state via an ISC process. The quantum efficiency for the ISC process depends on the radiative and nonradiative processes occurring from the lowest vibrational level of the first excited state.

$$Y_T = k_{ISC}/(k_{ISC} + k_f + \sum_i k_{i,f} + \sum_Q k_{Q,f} [Q])$$

where k_{ISC} is the unimolecular rate constant for the ISC process, k_f is the rate constant for fluorescence and $\sum_i k_{i,f}$ is the sum for the unimolecular nonradiative processes (except for ISC); $\sum_Q k_{Q,f} [Q]$ is the sum for all rate constants of bimolecular processes which result in the nonradiative deactivation of the singlet state. Substitution into the Y_P equation yields

$$Y_P = \left(\frac{k_{ISC}}{k_{ISC} + k_f + \sum_i k_{i,f} + \sum_Q k_{Q,f}[Q]}\right)\left(\frac{k_P}{k_P + \sum_i k_{i,P} + \sum_Q k_{Q,P}[Q]}\right)$$

The quantum efficiency of the phosphorescence is not only determined by structural properties of the compound, but also by environmental factors. In order to observe phosphorescence at room temperature when the excited triplet state is prompted to deactivate

through nonradiative means (e.g., collisional deactivation), it is necessary to provide a rigid medium for the phosphor. A rigidly-held molecule will have less probability to undergo radiationless transitions. Decreasing the rate constant for the nonradiative transitions, k_is, increases the quantum yield of the phosphorescence.

Similarly, Y_P can be greatly affected by the presence of quenchers. $\sum k_Q$ is a measure of all the quenchers present in the environment of the phosphor. Oxygen, for example, is a potent quenching agent of the excited triplet state (1,2,4-6); its presence can have a drastic effect on the phosphorescence.

Heavy Atom Effect

Formation of the triplet state depends on the quantum efficiency of the ISC process. This is known to be affected by the presence of heavy atoms, which can be directly bonded to the molecule ("internal heavy atom effect") or added to the microenvironment ("external heavy atom effect") (4). The spin-orbit coupling is directly proportional to the spin interaction energy, which for a hydrogen-like atom is proportional to Z^4, where Z is the atomic number (4) of the heavy atom. The addition of heavy atoms to the environment enhances the rate of ISC processes, increasing the quantum yield of the phosphorescence, decreasing the fluorescence quantum yield, and decreasing the phosphorescence lifetime (4). Several studies have been published on the origin of the "heavy atom effect" (3,7-10) and on its application to the RTP analysis of organic compounds of interest, including a variety of compounds of biological importance

(11-13), nonpolar polynuclear aromatic compounds (14,15), dyes (16), and polyaromatic hydrocarbons (17).

The heavy atom effect can affect both the radiative and the nonradiative singlet-triplet transitions; therefore, an increase in phosphorescence quantum yield does not always occur (4). Even when the triplet population has been increased, radiationless decay of the triplet may also increase and compete with phosphorescence. The specific path favored depends on the phosphor and the heavy atom in question (16). However, the radiative transitions seem to be enhanced more than the nonradiative ones (16).

The efficiency of the phosphorescence process then is greatly affected by the environment that surrounds the chromophore. The presence of a heavy atom enhancer, a rigid matrix, and the absence of quenchers (e.g., oxygen) are all important factors that will affect radiative emission from the triplet state.

Room Temperature Phosphorescence on Solid Supports

Types of Substrates Used for RTP

The use of solid substrates for the observation of phosphorescence at room temperature has attracted much attention in recent years. Although phosphorescence can, in principle, be observed from gases, liquids or solids, in practice the experimental conditions necessary for the observation of the radiative emission make the use of gases and liquids difficult. At room temperature the long-lived triplet molecule must be rigidly held to minimize the nonradiative processes and it must also be protected from the quenching effects of oxygen. The requirements of low pressures and presence of inert gases

in gaseous media make it impractical from the analytical point of view. The observation of phosphorescence in liquids is also difficult, since intermolecular collisional quenching contributes to weak or nondetectable phosphorescence and solutions must be thoroughly deoxygenated. The recent use of micelles or sensitizers have made solution RTP more useful for analytical applications (18). However, in order to obtain analytically useful RTP signals the micellar solutions must be thoroughly deoxygenated and the analyte must show appreciable solubility in the micellar environment (4). The lack of versatility of micellar RTP has limited its application as an analytical technique.

Phosphorescence was observed from solid matrices by immobilizing the phosphor in a frozen matrix, at the temperature of liquid nitrogen. The need for cryogenic equipment, the problems related to the introduction of the sample and matrix effects (cracking, snowing, nonuniformity in the microenvironment) made it an unpopular technique. Since the observation of phosphorescence at room temperature from compounds deposited on a solid surface for the first time by Roth in 1967 (19), solid substrate RTP has attracted much interest.

Several materials have been tested as substrates. At room temperature, phosphorescence has been observed from compounds adsorbed on filter paper, silica gel, aluminum oxide, sodium acetate, sucrose, starch, polymer-salt mixtures, chalk and other materials. Filter papers are the most commonly used substrates. Many compounds have been studied by use of various filter papers (4,6,20-22). A review

describing the applicability and limitations of cellulose supports follows this discussion.

Silica gel (23-26), sodium acetate (27-29), and polyacrylic acid-halide salt (26,30-32) mixtures were introduced by Hurtubise. Strong phosphorescence emission has been detected from polar or ionic compounds adsorbed on these substrates. Although several compounds that showed weak or no RTP signal when adsorbed on papers showed strong phosphorescence on silica gel or sodium acetate, the use of the latter is limited when compared to filter papers (14,17,33). Also, the limits of detection found for several hydroxyl-substituted aromatics in polyacrylic-halide salt mixtures was 2-15 times worse those found for filter paper (30).

Chalk and several inorganic materials (H_3BO_3, $CaHPO_4$, Na_2HPO_4, $CaSO_4$) containing at least one active site, such as carboxyl or a hydroxyl group, were investigated by Su and Winefordner (34). Plates were developed by blending certain amounts of the inorganic materials. The inorganic compound plates minimized background phosphorescence but did not enhance the analyte phosphorescence intensity enough to make them better substrates than filter paper.

Schulman and Parker (35) reported on the use of sucrose and starch for the RTP of sodium 1-naphthoate and sodium 4-biphenylcarboxylate. Other substrates from which phosphoresence has been observed are Nafion membranes (36), large-pore zeolites (37), and inorganic substrates such as asbestos and alumina (38). Reviews containing information on the different solid substrates used in RTP can be found in the literature (4,20,39).

Interactions Between the Phosphor and Substrate

After the initial reports of Roth (19) and Schulman and Walling (38,40) on RTP on solid substrates, many practical and theoretical studies have been published on the phosphorescence of different organic compounds deposited on a variety of supports. The phosphorescence properties of the lumiphors varied with the experimental conditions, that is, the type of support, solvent mixture, pH, presence of heavy atom enhancers, and the structural features of the phosphors (whether the compound is polar or nonpolar, ionic or neutral, heterocyclic or not). An understanding of the phosphorescence process and the mechanisms of interactions present under the different experimental conditions should lead to the development of an optimum substrate, an analytical model that will diminish or eliminate the uncertainties and/or the trial and error process involved in the selection of an adequate substrate for a given phosphor.

Several mechanisms of interaction have been postulated. Although the importance of the adsorbed state was initially emphasized (38,40), it was soon realized that the nature of the interactions was stronger than the one involved in a physisorbed state. The molecules need to be rigidly held on the surface in order to restrict the radiationless deactivation, therefore the weak dispersive forces that are present on the physisorbed state (4) probably are not strong enough to provide the rigidity necessary to observe phosphorescence. It is believed that hydrogen bonding is one of the predominant mechanisms of interaction present at the surface of the substrate, as supported by

several studies on different types of surfaces (5,23,24,27,29,32,33, 35,41,42).

Strong phosphorescence emission is observed from polar or ionized molecules when spotted from acidic or basic solutions onto the surface of a support containing active sites such as the hydroxyl groups of filter paper or silica gel chromatoplates (35,41). No, or extremely weak, phosphorescence is detected from nonpolar compounds even when these are spotted from acidic or basic solutions, as observed by Vo-Dinh et al. (33). These authors believe that only weak dispersion forces between the nonpolar molecule and the support are present and these are not sufficient to restrict the quenching of the phosphorescence. Wellons et al. (41) emphasized the importance of the ionic state of the analyte in producing RTP. The main interaction between an ionic or polar analyte and the support is attributed to hydrogen bonding of the ionized or polar functional groups to the hydroxyl groups of the support. The number of ionic sites in the molecule correlates with the relative intensity of the phosphorescence emission on filter paper. The strongest intensities were obtained from doubly-charged compounds when spotted from 1 M NaOH while uncharged or neutral species showed very weak emission (41). Schulman and Parker (35) observed considerable reduction in the RTP of sodium naphthoate when silanized paper was used as support as compared to nonsilanized paper. The reduction was attributed to a decrease in the amount of hydroxyl groups available at the surface of the silanized paper, compared to the nonsilanized paper. The authors also reported very weak or no phosphorescence from compounds incapable of hydrogen

bonding to the active sites of the paper. Likewise Ford and Hurtubise (23) observed that compounds that have potential for strong hydrogen bonding with silica gel chromatoplates exhibit more intense RTP than similar compounds that have fewer polar functional groups and, therefore, less hydrogen bonding ability. Hydrogen bonds seem to be also involved when sodium acetate is used as support. Von-Wandruszka and Hurtubise (27) postulated that anions of PABA hydrogen bonded to the carboxyl groups of the sodium acetate and are responsible for the strong phosphorescence observed when ethanolic solutions of PABA are spotted on this support.

Disruption of the hydrogen bonds formed at the surface of supports such as filter papers or silica has been postulated (5,35) as the probable cause of the quenching observed by moisture. Water molecules are capable of hydrogen bonding to the active sites of the paper or the silica gel surfaces and compete with the analyte for these sites. As a result, the analyte-support interactions diminish and the probability of radiationless deactivation increases, decreasing the phosphorescence emission. Water can also disrupt hydrogen bonding between cellulose fibers. This "softens" the matrix, allowing normal collisional deactivation to operate and enabling the transport of oxygen to the vicinity of the phosphor.

The presence of hydrogen bonds have been supported by UV diffuse reflectance, IR, multiple internal reflectance IR, and luminescence techniques. Dalterio and Hurtubise (32) studied the type of interactions of hydroxyl aromatic and aromatic hydrocarbons with polyacrylic acid-salt mixtures (PAA) and filter paper. The longest

wavelength absorption band of the selected compounds shifted to longer or shorter wavelengths depending on the nature of the hydrogen bond formed with the support. The IR shifts observed in the hydroxyl stretching band of PAA were taken as a measure of the degree of association of the hydroxyl groups with the adsorbed compounds. Likewise, shifts in the cellulose hydroxyl stretching band of filter paper, as observed from multiple internal reflectance spectra, indicated a net increase in the hydrogen bonding association of the filter paper hydroxyl groups. The authors concluded that hydrogen bonding interactions occurred between the model phosphors and the solid surfaces examined, and that they both behaved as proton donors, proton acceptors, or simultaneously as proton donors and acceptors.

Diffuse reflectance and IR spectroscopy has been used previously to study the type of interactions on silica gel (24) and on sodium acetate (27). Hydrogen bonding was postulated in both studies as the main type of interaction present. Silica gel containing a polymeric binder with carboxyl groups (such as polyacrylic acid) was found to be a very good support for inducing RTP from the model compound, benzo[f]quinoline (24). It was postulated that benzo[f]quinoline was adsorbed flatly on the surface with the carboxyl groups anchoring via hydrogen bonding with π electrons.

Reflectance maximum shifts of PABA on sodium acetate as compared to PABA on talc or starch (from which no RTP is detected) indicated strong interactions between the sodium acetate and PABA (27). Chemical interactions were believed to involve the formation of the sodium salt of PABA as well as hydrogen bonding.

Scharf and Winefordner (42) evaluated the spectral properties of p-aminoacetophenone (PAAP) in several matrices and at different temperatures. The fluorescence to phosphorescence ratio (F/P) of PAAP adsorbed on filter paper at -65°C and at room temperature was compared to the F/P obtained from PAAP dissolved in ethanol (a polar solvent) and in ether-pentane (EP) mixtures (a nonpolar solvent). The F/P of PAAP on filter paper at low temperature was much higher than in EP glass and similar to those observed in ethanol at 77 K. Based on these ratios and on the spectral characteristics of PAAP on different matrices the authors postulated that the interactions between PAAP and filter paper were similar to PAAP in ethanol at 77 K, namely hydrogen bonding interactions. The lifetimes of PAAP adsorbed on filter paper at room temperature and at -65°C were longer than the lifetimes of PAAP in nonpolar glass. This was taken as an indication of the strength by which PAAP molecules were held on the paper.

Senthilnathan and Hurtubise (29) used lifetime measurements to investigate the mechanism of interactions of PABA anion adsorbed on sodium acetate-sodium chloride mixtures. While the relative intensity of the phosphorescence emission increased with increased content of sodium acetate in the mixture, the lifetimes reached a maximum at 1.4% sodium acetate and remained fairly constant within the range of 1.4% to 100% sodium acetate. It was assumed by the authors that at 1.4% sodium acetate PABA had reacted with the maximum number of sodium acetate molecules which could be dissolved in the solvent during the "wet stage." A partial neutralization occurred and PABA was converted to its sodium salt and adsorbed on the sodium acetate surface. The

authors believed that for mixtures containing more than 1.4% sodium acetate, an increased rigidity of the matrix caused by increased undissolved sodium acetate content allowed for the observed increased intensity of the phosphorescence of PABA; a packing effect was believed to be operative.

Packing of the matrix is believed (6) to protect the phosphor from collisions with oxygen molecules and/or hold the phosphor in a more rigid state. This effect was initially reported by Niday and Seybold (5). These researchers observed an increase on the lifetimes of the RTP of 2-naphthalenesulfonate on filter paper packed with inorganic salts and sugars. The added compounds seemed to inhibit internal motion and decrease the nonradiative deactivation process. Alternatively, it is also possible that the added compounds "plug up" the channels and interstices of the matrix, thus decreasing permeability to oxygen. Both mechanisms could be operative.

Matrix effects which may or may not involve hydrogen bonding seem to play an important role in the phosphorescence process. McAleese and Dunlap (43) proposed a matrix isolation mechanism for the induction of solid surface RTP. It was based on the fact that cellulose fibers underwent considerable swelling in the presence of strong polar solvents such as water or ethanol. The swelling would allow penetration of the phosphor into the submicroscopic areas. During the drying process, the fibers lose the extra water molecules, collapse and trap the phosphors between the chains.

Packing and matrix effects were not the only mechanisms reported that did not involve hydrogen bonding. Lue Yen Bower and Winefordner

(17) studied the heavy atom effect on the RTP of several PAHs. Silver(I) enhances the phosphorescence of selected PAHs. It not only provides for an external heavy atom effect but also helps to create a rigid matrix for the phosphor. Silver(I) complexes with the π electron cloud of the aromatic hydrocarbons; it can also bond to the free hydroxyl groups of the cellulose. The silver ions then become a link, as the authors suggested, between the analyte molecule and the support, providing the necessary rigidity for the observation of phosphorescence.

Different theories on the mechanisms for the rigidly-held state of the phosphor have been formulated. Although we now have a better understanding of RTP as observed on solid substrates, there is still no fundamental analytical model. Additional studies are needed in order to develop the theoretical and experimental conditions of maximal solid-substrate RTP.

Evaluation of Cellulose Supports

Cellulose filter and chromatographic papers are the most commonly used substrates for RTP, as evidenced from the numerous publications on applications and fundamental aspects of RTP on this type of support as will be outlined below. They are simple and convenient to use, economical, readily available and very effective in inducing RTP from a variety of samples (4,6,20). The wide range of filter papers available with varied surface chemical and physical characteristics makes them especially attractive for the study of different types of phosphors.

Unfortunately, there are several disadvantages associated with their use in RTP. The cellulose filter papers, as all the other substrates which have been used for RTP, have a background phosphorescence (a broad featureless band from 400 to 600 nm). For a large number of organic substances emitting light in this spectral region, the phosphorescence detection limits increase (become poorer) depending on the magnitude of background signal. The background phosphorescence is also a source of spectral interference for weak phosphors. It has been attributed (44) to impurities present in the hemicellulose (and/or lignin) residues present in the cellulose fiber. Several attempts to reduce background phosphorescence have been reported in the literature. Treatments include soaking and chromatography with different solvents, and acidic and basic treatments (44,45), heating (45), sunlight bleach (44) and UV irradiation (45,46). The substances generating background emission are strongly absorbed or bound since the majority of the treatments fails to produce a reduction of the signal by more than 2-fold (44,45). However, McAleese and Dunlap (46) were able to achieve a 25-fold reduction in the background phosphorescence of Whatman 3MM chromatographic paper by illumination with white light from a Xenon lamp for 57 hr. The authors claim that by utilizing the illuminated support, the calculated detection limit for PABA was reduced to 0.5 pg, 200-20,000 lower than previously reported on solid supports (46).

There are other disadvantages associated with the use of filter papers. The rough and irregular surface of the filter paper is advantageous in that it offers some protection to the phosphors from

oxygen quenching (35,38); however, the numerous interstices between the fibers can be very detrimental. The fibers are opaque and may prevent the excitation light from reaching those molecules within the interstices, or the emitted light from reaching the detector. Penetration of the molecules into the bulk of the filter paper can be extensive. Together with penetration, there is also chromatographic migration of the species spotted on the surface. These factors affect the precision and accuracy of the analysis. The ideal substrate should allow the bulk of the analyte to remain on the surface.

Recently, Bateh and Winefordner (44) published an evaluation of cellulose as RTP substrate. They examined several cellulose pulps and filter papers to determine whether there is a successful combination of physical/chemical characteristics of these materials for RTP. They reported on DTPA (diethylenetriaminepentaacetic acid) treatment of S&S 903 filter paper. This chelating agent was used in an attempt to remove trace amounts of transition metals that might contribute to the background phosphorescence. The treatment did not produce the desired effect, but a considerable improvement in the analyte signal was observed compared with the untreated papers. This effect was attributed to the interaction of the DTPA with the analyte and to the filling of the gaps in the paper structure, which minimized absorption of analyte into the paper and provided for a more rigid matrix. The later effect is similar to the one proposed by Niday and Seybold (5) for filter papers packed with sugars and other inorganic substances.

The DTPA-treated S&S 903 was later compared with an anionic exchange paper Whatman DE-81 for the analysis of several model

compounds of drugs, PAHs, and pesticides (34). Whatman DE-81 contains, in addition to the hydroxyl groups bound to the cellulose, free anionic groups OH^- which were believed to provide for a more rigid matrix. Higher signals and lower background as compared with S&S 903 (treated and untreated) were observed for several drugs and pesticides in the presence of iodide as heavy atom enhancer. However, for PAHs, DE-81 with silver(I) or thallium(I) gave poorer detection limits. According to the authors, this was to be expected since DE-81 is an anionic exchange filter paper (a cation exchange filter paper was expected to be ideal for these compounds). In general, DE-81 was found to be a better substrate than DTPA-treated S&S 903.

Additional research is needed in this area so that improved or new substrates for RTP can be found that will provide a greater sensitivity, better precision, and lower limits of detection than present by available substrates.

CHAPTER 2
BIOCHEMICAL AND DRUG ANALYSIS BY ROOM TEMPERATURE PHOSPHORESCENCE

Applications of Room Temperature Phosphorescence to Compounds of Biomedical Interest

Room temperature phosphorescence on solid surfaces allows for a very simple, convenient and inexpensive method for detecting molecules on the surface of a solid support. Whether the support used is filter paper, a chromatoplate, or a pellet, the sample needs only to be spotted on its surface (with or without a heavy atom enhancer), dried, and measured under an inert atmosphere. The procedure requires a few minutes at most (depending on the time needed to evaporate the solvent, the drying procedure and/or the type of solvent used). Not more than a few microliters of sample are needed (usually 1 to 5 μL), this makes it especially attractive for biological, clinical or toxicological samples of which only a very small amount of sample is usually available.

Room temperature phosphorimetry is not only a simple and convenient method of analysis, but it is also very selective and sensitive. As any other luminescence technique, RTP provides the analyst with a choice of two wavelengths (excitation and emission) which gives it an advantage over absorption spectrometry, which involves the choice of only a single absorption wavelength. Since not all molecules luminesce and not all luminescent molecules are able to phosphoresce, phosphorimetry becomes a very selective technique. The

phosphorescence properties of a molecule spotted on a solid substrate (as shown in chapter 3) are greatly affected by the microenvironment that surrounds the lumiphor. By the appropriate selection of the experimental conditions (e.g., support, solvent mixture, pH, and heavy atom enhancer), it is possible to increase the phosphorescence quantum yields of a compound and measure it in the presence of other nonphosphorescent or weakly phosphorescent molecules. The selectivity inherent in the method has made it possible to resolve components of a mixture without prior chromatographic separation. It was used, for example, by Bateh and Winefordner (47-49) for the determination of drugs in their pharmaceutical formulations without prior separation from other compounds present in the formulations. Using sodium acetate as support, Von-Wandruszka and Hurtubise (50) developed an assay for PABA in vitamin tablets. The method was so specific for PABA (and not for the other ingredients present in the tablets) that the tablet extract exhibited an RTP spectrum identical to those of pure PABA samples. Su et al. (51) used RTP for the analysis of synthetic mixtures of phosphorescent pesticides. A combination of substrate and heavy atom effects was used to resolve the RTP responses of the pesticides in their mixture (51). The same effects have been used by Asafu-Adjaye et al. (52) for the multicomponent analysis of mixtures prepared from four of five analytical reference standards of toxic substances.

Room temperature phosphorescence could be especially suitable in clinical chemistry. Since there are only a few phosphorescent interferences in serum (53), RTP can be used for the determination of

drugs and alkaloids in blood without tedious pretreatment procedures. Its sensitivity allows the analyst to reach the limits of detection needed to measure the compounds of interest. It was possible, for example, to measure the concentration of salicylic acid in blood serum by RTP at concentration levels 10-fold lower than is conventionally possible (54). The linear range for the RTP analysis of salicylic acid extended from 0.0 to 400 ng.

Limits of detection in the subnanogram and namogram region have been obtained from the RTP analysis of aromatic compounds of biomedical interest (e.g., drugs, pharmaceuticals, peptides, etc.). Jakovljevic (55) detected cinoxacin, a potent antimicrobial drug at picogram levels on filter paper by RTP. De Lima and Nicola (56) investigated the RTP of nalidixic acid, an antimicrobial drug for control of chicken infections. Vo-Dinh et al. (12) were able to observe phosphorescence of PABA, salicylic acid, cocaine, and barbituric acid at the nanogram and subnanogram levels. Phosphorimetry on filter paper proved to be a very sensitive and simple method for the analysis of several pharmaceuticals (47-49,57). The spectral characteristics and the analytical conditions which allow the observation of phosphorescence from these compounds spotted on solid substrates are well documented in the literature. The emphasis has been on obtaining important analytical data, that is, the figures of merit for the analysis: reproducibility, sensitivity, limits of detection, and useful analytical range. These data are important in order to

demonstrate the potential applicability of the technique to real-life samples.

In general, RTP can be considered a suitable method of analysis for compounds of biochemical, clinical, toxicological or medicinal interest; it is simple, convenient, selective and sensitive, and requires a small amount of sample. There are, however, very few applications found in the literature to real-life samples (47-50,54,56,58). It is one of the objectives of this research to observe the RTP spectral characteristics of several compounds of biomedical interest and to obtain analytical data which will show the applicability of room temperature phosphorescence to the analysis of the compounds. The compounds studied were several indoles, including 3-carbinolindole, 5-methoxyindole, 3-methylindole, 3-indoleacetic acid and DL-β-3-indolelactic acid, and diazepam.

Room Temperature Phosphorescence Analysis of Indole Derivatives

Introduction

The luminescence properties of indoles have long been of interest, mainly because the indole ring constitutes the aromatic moiety of tryptophan, an important biomolecule which can act as an intrinsic fluorescence probe of proteins and enzyme structures (59). The low temperature phosphorescence (LTP) properties of tryptophan were used by Koppa et al. (60) to identify and characterize nucleic cell components and by Vladimirov and Litrin (61) to determine human serum and egg albumin. Low temperature phosphorescence has been used also for the determination of other indole derivatives with much success (62,63). Room temperature phosphorescence does not require

the use of cryogenic equipment and thus does not have the problems associated with the frozen matrices of LTP; assays of the indole derivatives based on RTP could provide alternative, simpler methods for the existing bioassays.

Apparatus

All RTP spectra were obtained with a Perkin-Elmer LS-5 fluorescence spectrophotometer, interfaced with a Perkin-Elmer CLS-3600 luminescence data station computer. The LS-5 is fitted with a Xe flash lamp pulsed at the line frequency. The photomultiplier signal is measured with gated electronics. The start of the gate (t_d) and the duration of the gate (t_g) can be selected in multiples of 10 μs. A gate time and delay time of 9.0 and 0.3 ms, respectively, were used for the collection of the RTP spectra of the indoles. The scanning of excitation and emission spectra (scan speed 120 nm/min) and the evaluation of peak intensity and wavelength were processor-controlled by the application program PECLS. Excitation and emission slits widths were both set at the same bandpass value of 10 nm.

An aluminum sample holder was constructed (Fig. 2) to fit the standard Perkin-Elmer room temperature fluorescence multiple sampling compartment. Four identical sample holders could be placed simultaneously in the sample compartment which minimized the drying time. Better precision was obtained by performing all the measurements from the same prelabelled position of the standard fluorescence compartment.

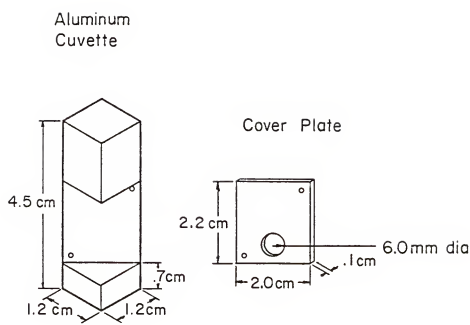


Fig. 2. Room temperature phosphorescence sample holder constructed to fit the standard Perkin-Elmer fluorescence multiple sampling compartment.

The phosphorescence intensities were measured with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD). The spectrophotofluorometer is fitted with an Aminco 150 W Xenon arc lamp, a potted Hamamatsu 1P21 photomultiplier tube (Hamamatsu Corp., Middlesex, NJ), a MFE Plomatic FIS X-Y recorder (MFE Corp., Salem, NJ), and a laboratory-constructed phosphoroscope for use with the multiple sampling bar system (64).

Chemicals

The indoles studied were indole, 3-carbinolindole, 5-cyanoindole, 5-fluoroindole, 5-methoxyindole, 2-methylindole, 3-methylindole, 5-nitroindole, 3-indoleacetic acid, 3-indolebutyric acid, DL-β-3-indolelactic acid, 3-indoleacrylic acid, 3-indolepyruvic acid monohydrate, and 3-indoxyl acetate. Potassium iodide was used as heavy atom enhancer. The solvents used to prepare solutions were absolute ethanol and "nanopure" demineralized water. Whatman DE-81 filter paper was used as support for RTP of the indoles (a description of this paper can be found in chapter 3). Table 1 contains a list of the suppliers/manufacturers for all the reagents and materials used.

Procedures

Measurement procedure. Portions of a KI solution (2 μL were used for indolecarboxylic acids, 1 μL for the remaining indoles) and a sample solution aliquot (2 μL) were successively introduced by means of a micropipetter (SMI, Emeryville, CA) onto 0.25 in diameter filter paper disks which were held in one of the sample holders. Immediately afterwards, the sample holder (or sampling bar) was inserted into the sample compartment of the spectrophotofluorimeter where the samples

Table 1. List of Reagents and Materials.

Reagents/Materials	Manufacturers/Suppliers
absolute ethanol	Florida Distillers Co. (Lake Alfred, FL)
benzene	Fisher Scientific Co. (Fair Lawn, NJ)
caffeine	Sigma Chemical Co. (St. Louis, MO)
carbaryl	Environmental Protection Agency (USA)
3-carbinolindole	Aldrich Chemical Co. (Milwaukee, WI)
cetyltrimethylammonium-bromide	"
5-cyanoindole	"
diazepam	Sigma Chemical Co. (St. Louis, MO)
diethylenetriaminepenta-acetic acid	"
3,5-diiodotyrosine	Nutritional Biochemicals Co. (Cleveland, OH)
5-fluoroindole	Sigma Chemical Co. (St. Louis, MO)
hydrochloric acid	Fisher Scientific Co. (Fair Lawn, NJ)
5-hydroxytryptophan	Nutritional Biochemicals Co. (Cleveland, OH)
indole	Aldrich Chemical Co. (Milwaukee, WI)
3-indoleacetic acid	"
3-indoleacrylic acid	"
3-indolebutyric acid	"
DL-β-3-indolelactic acid	"
3-indolepyruvic acid monohydrate	"
3-indoxyl acetate	"
isopropanol	Fisher Scientific Co. (Fair Lawn, NJ)
lead acetate	"
lead nitrate	"
mercuric chloride	"
methanol	American Burdick & Jackson (Muskegan, MI)
5-methoxyindole	Sigma Chemical Co. (St. Louis, MO)
2-methylindole	"
3-methylindole	"
5-nitroindole	Aldrich Chemical Co. (Milwaukee, WI)
potassium iodide	Fisher Scientific Co. (Fair Lawn, NJ)
potassium tetrachloro-platinate (II)	Strem Chemicals Inc. (Newburyport, MA)
8-quinolinol	Eastman Organic Chemicals (Rochester, NY)
serum (human) (Validate)	General Diagnostics (Morris Plains, NJ)
silver nitrate	Mallinckrodt (St. Louis, MO)
sodium dodecyl sulfate	Fisher Scientific Co. (Fair Lawn, NJ)
sodium hydroxide	"
S&S 903	Schleicher & Schuell Inc. (Keene, NH)
sulfuric acid	Fisher Scientific Co. (Fair Lawn, NJ)
thallous nitrate	PCR Inc. (Gainesville, FL)
theophylline	Sigma Chemical Co. (St. Louis, MO)
Whatman CM-23 (carboxy-methylcellulose	Whatman Laboratory Products Inc. (Clifton, NJ)
Whatman DE-81	"
Whatman No. 1	"
Whatman P-81	"
water (nanopure, demineralized)	Barnstead System of Sybron (Boston, MA)

were allowed to dry for about 8 min under a flow of nitrogen. RTP measurements were then performed.

Standard preparation. The standard stock solutions of the indoles were made by dissolving in ethanol an accurately weighed portion of the compound of interest. Standard solutions were prepared by appropriate dilutions of the stock solution using ethanol for indole, 3-carbinolindole, 5-cyanoindole, 3-fluoroindole, 5-methoxyindole, 2-methyl, and 3-methylindole or an ethanol-water mixture (50:50 v/v) for the indolecarboxylic acids.

Results

RTP spectral characteristics. All the indole derivatives under study showed RTP spectra when adsorbed on DE-81 filter paper in the presence of 1 or 2 μL of 1 M KI, with the exception of 5-nitroindole, 3-indoxyl acetate, and 3-indoleacrylic acid; no detectable phosphorescence signal can be observed from 2 μL of 10^{-3} M solutions of these indoles. The RTP excitation and emission wavelengths are presented in Table 2.

The excitation and emission spectra of the indole carboxylic acids (Fig. 3a) (as measured on the Aminco-Bowman) are very similar. They present broad bands with little vibrational fine structure, as previously observed with other compounds (4). The rest of the indole derivatives (as measured with the LS-5) present spectra that are blue shifted with respect to the indolecarboxylic acids. In general, the room temperature phosphorescence spectra of several of the indoles included in this study are very similar to their low temperature phosphorescence spectra reported in the literature (65).

Table 2. Room Temperature Phosphorescence Spectral Properties of Indoles.

Compound[a]	λ_{ex}, nm[d]	λ_{em}, nm[d]
Indole[b]	(261), 270, (278)	(410), <u>430</u>, 450
3-Carbinolindole[b]	(245), (259), 279	(419), 440
5-Cyanoindole[b]	243, 258, (272), <u>281</u>	(421), 439
5-Fluoroindole[b]	(261), <u>269</u>, 278	(412), 434, (452)
5-Methoxyindole[b]	235, (256), <u>268</u>, (278)	(419), 437
2-Methylindole[b]	(259), 269, (277)	428
3-Methylindole[b]	282	(422), <u>442</u>, 467
3-Indoleacetic acid[c]	286	(429), 450
3-Indolebutyric acid[c]	287	448
2-Indolecarboxylic acid[c]	299	(475), 490
DL-3-Indolelactic acid[c]	288	448
3-Indolepyruvic acid[c]	283	442

[a] Concentrations are approx. 10^{-3} M.
[b] Ethanol is used as solvent; measurements are done with LS-5 Perkin Elmer Spectrophotofluorometer.
[c] A water:ethanol (50:50 v/v) mixture is used as solvent; RTP spectra are obtained with Aminco-Bowman spectrophotofluorometer.
[d] Wavelengths of the main peaks are underlined (when there are several components); wavelengths of shoulders are given in parentheses; precision of wavelength value ± 1 nm.

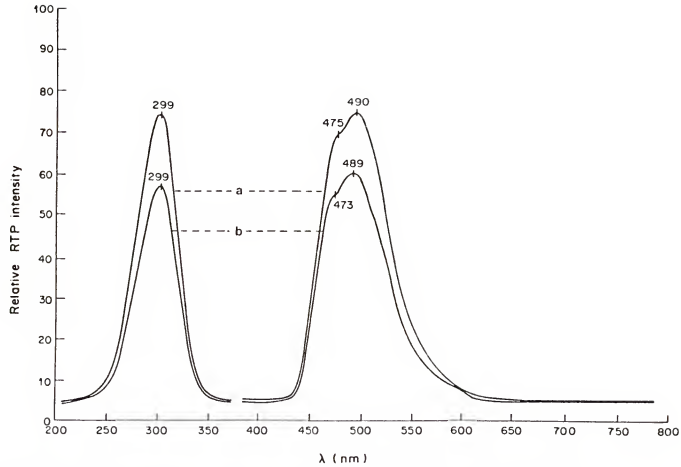


Fig. 3. Room temperature phosphorescence spectra of 2-indolecarboxylic acid (10^{-3} M) on DE-81 filter paper, in the presence of 1 M KI.
a. Excitation and emission spectrum of neutral water:ethanol (50:50 v/v) solution
b. Excitation and emission spectra of basic (0.5 M NaOH) water:ethanol (50:50 v/v) solution

Analytical figures of merit. Strong phosphorescence emission can be observed from the indole compounds spotted on DE-81 and in the presence of 1 or 2 µL of 1 M KI, with the exception of 5-nitroindole, 3-indoleacrylic acid, and 3-indoxyl acetate which did not phosphoresce under the experimental conditions used, and 3-indolepyruvic acid, whose signal was relatively weak compared to the rest of the indoles. The characteristics of the log-log calibration curves and the limits of detection (LOD) are presented in Table 3. The useful analytical ranges (UAR) for the analysis of the compounds are relatively large, between 30 and 200 concentration units. The slopes of the log-log calibration curves vary from 0.89 to 1.04, and the precision is in most cases satisfactory, as shown by the correlation coefficients. The LODs are particularly low, ranging from 0.1 to 2.1 µg/mL depending on the compound tested. The absolute LOD of 0.4 ng (corresponding to a concentration of 0.2 µg/mL) for 3-indoleacetic acid, compares favorably with the literature values of 50 ng obtained by silica-gel thin-layer fluorimetry (66), 0.02 µg/mL evaluated by LTP (64), and 0.1 ng determined by HPLC with fluorescence detection (67). These results demonstrate that RTP constitutes a precise, simple, and sensitive analytical method for the determination of the indoles, including the indolecarboxylic acids.

Room Temperature Phosphorescence of Diazepam and Its Application to the Determination of Diazepam in Serum and in Pharmaceutical Formulation

Introduction

Diazepam is widely prescribed as a mild tranquilizer or hypnotic (68). It is also among the most frequently encountered drugs in

Table 3. Analytical Figures of Merit of Indoles Evaluated on DE-81 With 1 M Indole.

Compound	UAR[a]	Slope[b]	Correlation Coefficient	LOD[c] (µg/mL)	Absolute LOD[d] (ng)
5-Cyanoindole	30	0.89	0.998	0.51	1.0
5-Fluoroindole	50	1.04	0.998	2.1	4.2
5-Methoxyindole	100	0.90	0.990	1.5	3.0
2-Methylindole	140	0.93	0.991	1.1	2.2
3-Methylindole	200	0.89	0.997	1.0	2.0
3-Indoleacetic acid	100	0.98	0.995	0.2	0.4
3-Indolebutyric acid	200	0.94	0.998	0.5	1.0
DL-ß-3-Indolelactic acid	100	1.01	0.999	0.2	0.4
2-Indolecarboxylic acid	150	0.95	0.999	0.1	0.2

[a] Useful analytical ranges, corresponding to the ratio of the upper concentration of linearity (within 5%) and the limit of detection.
[b] Slope calculated from log-log calibration plot.
[c] Limit of detection, defined as the concentration of the spotted solution giving a signal-noise ratio of 3.
[d] Calculated for a 2 µL sample solution.

postmortem and emergency toxicological analyses (69). Several methods for the qualitative and quantitative determination of diazepam in biological fluids have been reported (70-77). Analytical methods include GC (70,71), HPLC (72,73), TLC (74), radioreceptor (75), UV (76), and enzyme immunoassay (77). The majority of these methods requires substantial sample preparation. A rapid, sensitive screening method for diazepam determination in biological fluids is very desirable and would be most helpful to the analytical toxicologist. In this research, the use of solid substrate RTP is evaluated as a rapid, simple and sensitive method for toxic levels of diazepam in serum.

Room temperature phosphorimetry has already been used successfully for the analysis of drugs in pharmaceutical formulations (47-49). The selectivity inherent to RTP has allowed the determination of pharmaceuticals in a variety of matrices. Since the patent on Valium expired recently, generic brands of diazepam will soon become available. Quality control of manufactured formulations requires a selective and rapid procedure for the determination of the active ingredient in a variety of matrices. In the present work, RTP is evaluated as a quantitative method for diazepam assay in tablets.

The RTP of diazepam is observed under optimum experimental conditions from tablet extractions and from diazepam-spiked blood serum samples and their benzene extracts. Analytical figures of merit of the technique are determined using Whatman No. 1 filter paper under optimal pH and heavy atom conditions.

Apparatus

All RTP spectra and intensity measurements were performed with the Perkin-Elmer LS-5 fluorescence spectrometer (described in the previous section). A delay time of 0.03 ms and a gate time of 9.0 ms were chosen so as to maintain the ratio of analyte intensity to background intensity as high as possible. A 305 nm cut-off filter was used on the emission side at all times. Bandwidth slits of 10 nm and 5 nm were used on the excitation and emission side, respectively. A laboratory constructed sample compartment (78) was attached to the spectrometer and this allowed the use of a home-built sample rod and holder (78). Whatman No. 1 filter paper disks of 10 mm diameter were used.

Reagents

The chemicals used for the RTP analysis of diazepam were mercuric chloride (used as heavy atom enhancer), nanopure deionized water, absolute ethanol, methanol, benzene, hydrochloric acid, and sulfuric acid (used in the preparations of the different solvent systems). Lyophilized human serum and Valium tablets were used for the applications of the RTP determination of diazepam. A list of the manufacturers/suppliers for the reagents and materials used can be found in Table 1.

Standard and Sample Preparation

A standard stock solution (1000 µg/mL) of diazepam was made by dissolving in ethanol an accurately weighed portion of diazepam. Standard solutions were prepared daily as needed by appropriate

dilutions of the stock solution with ethanol. An acidic solution of the heavy atom $HgCl_2$ (0.1 M) was prepared with 0.1 M HCl in ethanol:water (50:50 v/v).

Valium tablets. Valium tablets (containing 10 mg of diazepam per tablet) were prepared for assay by dissolution-dilution with 0.05 M H_2SO_4 in methanol. For the analysis of representative samples, 16 tablets were weighed together, powdered with mortar and pestle, and four portions were dissolved in 50 mL of the solvent system to give solutions of nominally 100 µg/mL in diazepam. Appropriate dilutions were made with the same solvent to yield sample solutions containing about 10 µg/mL of active ingredient. Standard solutions of 4, 10, 20, and 25 µg/mL of diazepam used for the analysis of the tablet dissolutions were prepared in 0.05 M H_2SO_4 in methanol.

Serum samples. Serum samples containing known amounts of diazepam were prepared for the assay. Appropriate volumes of diazepam standard solution (in ethanol) were pipetted into individual tubes and evaporated to dryness under nitrogen at room temperature. Aliquots of reconstituted human serum were subsequently added to the tubes. Serum samples containing 1.0, 5.0, 10, 20, 100, and 200 µg/mL of diazepam were prepared.

Extractions of diazepam from serum samples were made by addition of an equal volume of benzene followed by mixing for 1 min and centrifugation at 3000 rpm for 5 min to effect layer separation. The organic layer was then sampled for RTP analysis. Standards of diazepam were prepared by pipetting known volumes of diazepam standard ethanolic solutions into test tubes, evaporating to dryness and adding

known volumes of benzene extracts of drug-free serum. Standards containing 1.0, 5.0, 10, 20 and 50 μg/mL of diazepam were prepared.

Procedure

Valium tablet assay. The RTP of the four dilution samples of diazepam tablets (Valium) (containing 10 μg/mL of active ingredient) was observed in the presence of 0.1 M $HgCl_2$ in ethanol:water (50:50 v/v). The excitation and emission wavelengths were 300 and 469 nm, respectively. Sixteen measurements were carried out for each sample and standard.

Serum assay. The spiked serum samples, their benzene extracts and the diazepam standards (in benzene extracts of drug-free serum) were measured in the presence of an acidic solution of mercuric chloride (0.1 M $HgCl_2$ and 0.1 M HCl) as the heavy atom solution. The excitation and emission wavelengths maxima under these conditions were 290 and 464 nm, respectively.

Measurement process. Filter paper disks were placed under the cover plate of the sample holder and the cover plate tightened into place on the holder with two screws. A 3 μL heavy atom solution followed by a 3 μL sample solution were spotted onto the paper disks by means of an SMI micropipetter. Immediately afterwards, the sample rod containing the paper disks was inserted into the sample compartment of the spectrometer where the samples were allowed to dry for 15 min under a flow of nitrogen. Phosphorescent measurements were then performed.

Results and Discussion

RTP spectral properties. The RTP excitation and emission spectra of ethanolic solutions of diazepam spotted on the surface of Whatman No. 1 and in the presence of 0.1 M $HgCl_2$ are shown in Fig. 4. The broad, structureless emission band has a maximum at 469 nm. Two bands with maxima at 306 and 389 nm can be seen in the excitation spectrum.

Analytical figures of merit. The RTP calibration curves and limits of detection of diazepam standards prepared in different solvent systems and under optimal experimental conditions are presented in Table 4. Analytical curves were obtained with a sensitivity that varied depending on the solvent system used and the experimental conditions (see note "e" of Table 4). Higher intensities and, as a result, higher sensitivity were obtained from diazepam dilutions in 0.05 M H_2SO_4 in methanol, the solvent system used for the dissolution-dilution of diazepam tablets. The RTP absolute limits of detection are particularly low, ranging from 0.5 to 1.8 ng, depending on the experimental conditions. A useful analytical range varying from 80 to 100 concentration units was obtained. These results indicated that RTP could be used for the quantitative analysis of diazepam.

Pharmaceutical formulation. The diazepam concentration of tablets containing 10 mg of active ingredient was determined by comparing the relative phosphorescence intensity of the tablet dissolutions with the diazepam standards in the same solvent system.

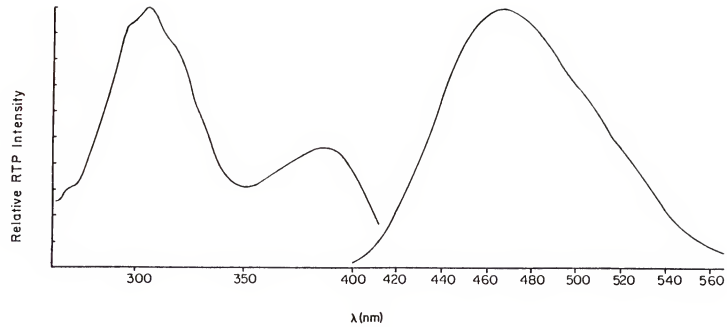


Fig. 4. Room temperature phosphorescence spectrum of diazepam ($1.2X10^{-3}$ M in ethanol) on Whatman No. 1 in the presence of 0.1 M $HgCl_2$.

Table 4. Room Temperature Phosphorescence Analytical Figures of Merit for Diazepam in Different Solvent Systems.

Solvent System	Slope[a]	Correlation Coefficient	UAR[b]	LOD[c] (μg/mL)	Absolute LOD[d] (ng)
Ethanol	6.5	0.999	80	0.6	1.8
0.05 M H_2SO_4 in Methanol[e]	19.1	0.999	100	0.2	0.6
Benzene-Serum Extracts	9.4	0.999	100	0.5	1.5

[a] Slope of analytical curve evaluated on Whatman No. 1 filter paper in the presence of 0.1 M $HgCl_2$. When ethanol and benzene serum were used as solvents, the $HgCl_2$ solution was made 0.1 M in HCl. For diazepam in 0.05 M H_2SO_4-methanol, a neutral solution of $HgCl_2$ was used.

[b] UAR is the useful analytical range, corresponding to the ratio of the upper concentration of linearity and the limit of detection.

[c] LOD is the limit of detection.

[d] Absolute LOD was calculated for a 3 μL sample solution.

[e] A new Xe lamp was installed prior to the determination of diazepam in 0.05 M H_2SO_4-methanol. The higher intensity obtained from the new lamp probably contributed to the higher sensitivities obtained in the experiment.

Linear regression analysis of the RTP intensities of the standard solutions gave the line RTP Intensity = 19.1 (μg/mL of Diazepam) + 3 with a correlation coefficient of 0.9999. The useful analytical range for the analysis extended from 0.2 to 20 μg/mL.

The mean concentration found experimentally for 16 measurements of each of four samples (equivalent to 10.0 μg/mL of active ingredient) was 10.2 μg/mL. They ranged from 9.8 to 10.6 μg/mL with a relative standard deviation of 3.4%. The good recoveries obtained from the assay show that RTP can be successfully applied to the quantitative determination of diazepam in pharmaceutical formulation.

Blood serum. Serum samples containing known amounts of diazepam were directly analyzed by measuring the phosphorescence intensities of the spiked serum excited at the excitation maximum wavelength (290 nm). The RTP emission intensities of the serum samples under optimal experimental conditions are tabulated in Table 5. Without extraction of diazepam, no phosphorescence could be detected above background phosphorescence for serum samples containing less than 100 μg/mL of diazepam (see Table 5). Diazepam is highly bound to plasma proteins; reported values according to different methods range from 96 to 99% (79). The lower sensitivity of diazepam in serum as compared to that of diazepam solutions in organic solvents (see Table 4) seems to indicate that the binding of diazepam to serum albumin appreciably affects its phosphorescence properties. If we consider that only 1 to 4% of the total diazepam amount in serum is free from albumin binding, then the results suggest that only free diazepam phosphoresces in unextracted serum. The observed RTP intensity corresponds to a

Table 5. Emission Intensity of RTP of Serum Samples Spiked With Diazepam Before and After Extraction.

Diazepam Concentration (μg/mL)	Net RTP Intensities[a,b] of Serum Samples		Net RTP Intensities of Diazepam Stds.
	Before Extraction	After Extraction	
1.0	-	9	10
5.0	-	47	46
10.0	-	101	90
100.0	12	AAUR[c]	-
200.0	36	AAUR	-

[a] Evaluated on Whatman No. 1 filter paper; 0.1 M $HgCl_2$ in 0.1 M HCl methanol:water (50:50 V:V) used as heavy atom solution.

[b] The Net Relative Intensities have been background substrated. Average of six measurements given. RSD = 15%.

[c] AAUR: Above Analytically Useful Range of Analytical Curve.

diazepam concentration within the range of 1 to 4% of the total amount added.

Extraction of diazepam from the serum was accomplished with benzene. Physical separation of the organic layer was not necessary. The organic layer was sampled directly and the RTP intensity of diazepam was measured at the maximum excitation and emission wavelengths, 290 and 464 nm, respectively, and under optimal experimental conditions. Results are reported in Table 5. The intensities obtained from the benzene extracts of the spiked serum are similar to those obtained from standard diazepam solutions prepared with extracts of drug-free serum, indicating that good recoveries are possible with a single extraction step. Co-extracted compounds did not interfere with the determination as can be seen by comparing the RTP characteristics of diazepam in ethanol with those in benzene extracts of the serum (see Table 4).

Serum solutions of diazepam in benzene extracts gave a linear calibration curve RTP Intensity = 9.43 (μg/mL of Diazepam) - 1, obtained through linear regression analysis with a correlation coefficient of 0.999. The LOD for the analysis is 0.5 μg/mL. Although the LOD is relatively high for monitoring diazepam in blood serum at therapeutic levels [0.3-0.5 μg/mL (80)], it is good enough to allow the analysis of diazepam in serum at toxic levels [3-14 μg/mL (80)].

Room temperature phosphorimetry offers a simple, selective, and sensitive method for the analysis of diazepam both in blood serum (at toxic levels) and in tablet formulation.

CHAPTER 3
EFFECTS OF THE MICROENVIRONMENT ON THE ROOM TEMPERATURE PHOSPHORESCENCE OF COMPOUNDS OF BIOLOGICAL INTEREST

Environmental Conditions

The RTP properties of compounds observed from the surface of filter papers is mostly determined by the microenvironment that surrounds the phosphor. Based on the proposed theories of RTP, the matrix must provide the necessary rigidity that reduces radiationless deactivation. A rigid matrix is achieved through the support-heavy atom-phosphor interactions present at the surface in the "dry stage." These interactions depend largely on the chemistry occurring at the wet surface at the moment of spotting.

Several factors, such as the analyte structural features, the pH of the solvent, the type of support, and the kind and amount of heavy atom present, affect the reactions or interactions occurring at the "wet stage." The presence of ionizable or polar groups in the phosphor are known to favor the RTP process. It is believed (1-6) that chemical interactions between these groups and the active sites of the support are responsible for providing the desired rigidity (see chapter 1). The chemistry of the phosphor in solution, prior to the spotting step must also be considered. The experimental conditions (solvent mixture, pH) can be selected so as to allow a single species of the compound, or a combination of them to be present in solution, which then interacts chemically with the active sites of the

surface. For example, no RTP is observed from 2-naphthol spotted on paper from neutral solutions, but intense RTP is detected for 2-naphthol in 1 M NaOH solutions where only the anionic species, sodium 2-naphthoxide, is present (6). Ramasamy and Hurtubise (81) found that, in 0.1 M HBr and 0.1 M HCl, the RTP signal of several nitrogen heterocyclics and aromatic amines deposited on filter paper was much stronger than that obtained in neutral media and alkaline solutions. Hurtubise and Smith (26) studied the pH effect on the RTP of several aromatic carboxylic acids adsorbed on silica gel and polyacrylic acid-sodium chloride mixtures. They found that terephthalic acid and coumarin-3-carboxylic acid adsorbed on silica gel chromatoplates containing a polyacrylate salt, yielded stronger RTP signals when sorbed from hydrochloric acid than from neutral solutions and concluded that interactions between the undissociated form of the aromatic carboxylic acid and the polyacrylate were responsible for inducing the phosphorescence.

The solvent mixture not only influences the chemistry of the compound, but also plays an important role at the wet surface. It may interact with the fixed or free groups associated with the type of support used. A considerable amount of water can be absorbed by the cellulose fibers due to formation of hydrogen bonds between the hydroxyl groups of the fiber and water molecules (82). The swelling of the fibers in the presence of polar solvents such as water or ethanol is the basis of the matrix isolation mechanism (43) mentioned previously (see chapter 1). Other types of interactions may be present. For example, neutralization reactions have been postulated

for polyacrylic acid-halide salts mixtures (83) and sodium acetate (27) substrates when phosphors are spotted from acidic or basic solvents.

Heavy atoms are usually added to the environment to enhance the phosphorescence process. These atoms play an active role at the surface since they not only interact with the phosphor, which makes possible the increased rate in the ISC processes and the enhancement of the phosphorescence, but they may also interact with the active sites of the support, as proposed by Lue Yen Bower and Winefordner (17). The magnitude of the heavy atom effect has been shown to vary considerably in RTP, depending on the kind and amount of heavy atom used and on the particular phosphorescence compound (16,17,34).

The conditions at the surface must favor a strong interaction between the support and the phosphor. These are not always easy to predict or elucidate. In this research, the effects of the support, pH, heavy atom and structural features are observed. An attempt is made to elucidate the mechanisms of interactions which prevail for the selected compounds under different experimental conditions.

Study of the RTP of Indole and Several of Its Derivatives

The study of the environmental effects on the RTP properties of several indole compounds was divided into two parts. The influence of the type of filter paper, the heavy atom effect of Tl(I) and I^-, and the substituent effect on the RTP spectra and intensities of indole, 3-carbinolindole, 5-cyanoindole, 5-fluoroindole, 5-methoxyindole, and 2-methylindole were observed in the first part of this study. The second part evaluates the pH and substrate effects on the RTP of

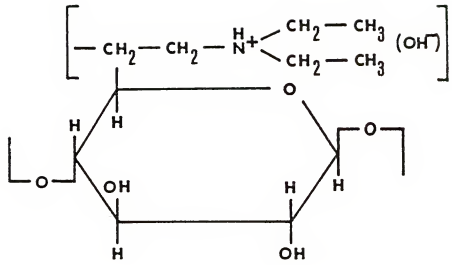
several indolecarboxylic acids: 3-indoleacetic acid, 3-indolebutyric acid, DL-3-indolelactic acid, 3-indoleacyrlic acid, 3-indolepyruvic acid monohydrate, and 3-indoxylacetate.

Effects of Substrate and of Heavy Atom on the RTP of Several Indoles

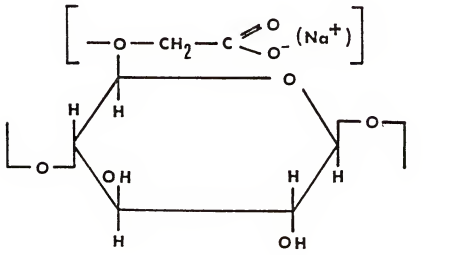
Introduction

Three types of substrate were used for the indole study: the anion exchange filter paper (Whatman DE-81), a cellulose paper (S&S 903) treated with diethylenetriaminepentaacetic acid (DTPA), and a cation exchange resin (Whatman CM 23 carboxymethylcellulose resin). Whatman DE-81 filter paper has free OH^- as well as $-NH(CH_2CH_3)_2^+$ functional groups attached to the cellulose polymer (Fig. 5a). The use of ion-exchange filter paper for RTP was proposed recently by Su and Winefordner (34). They found that DE-81 decreased significantly the limits of detection of some pesticides and drugs, and minimized the background phosphorescence compared to other filter papers. The authors believe that the presence of ionic exchange groups, in addition to the hydroxyl groups in the substrate, holds ionic compounds even more rigidly than substrates which do not contain ionic exchange groups.

Filter paper DTPA-treated S&S 903 was used previously by Bateh and Winefordner (44) for the analysis of several drugs. The chelating agent DTPA was believed to fill in the gaps in the paper and improve the adsorption of the molecule on the substrate. It could also provide for additional sites of interaction with the phosphor (see discussion of supports in chapter 1).



DIETHYLAMINOETHYL CELLULOSE (ANION)



CARBOXYMETHYL CELLULOSE (CATION)

Fig. 5. Structures of the anion filter paper DE-81 (a) and of the cation CM 23 resin (b). Structures taken from reference 34 (with permission of the authors).

No cation exchange filter paper was available at the time of the indole study. In order to compare the effect of a cation exchanger in the presence of both negatively and positively charged heavy atom enhancers, a cation exchange resin CM 23 was used. The regenerated resin contains bonded carboxylate groups (Fig. 5b).

Tryptophan and unsubstituted indole exhibit strong RTP on filter paper in the presence of NaI (12,13). Meyers and Seybold (13) found that the RTP of tryptophan was enhanced 340-fold and that of indole itself 370-fold by addition of 1.0 M NaI. The heavy atom effect of Tl(I) and I^- deposited on different supports on the RTP signal is evaluated. In addition, an attempt is made to study the substituent effect on the RTP spectra and intensities of indoles.

Experimental

The apparatus and procedures were described in chapter 2 (Experimental Section). Table 1 contains a list of the suppliers for all the reagents used in the study. The DTPA-treated S&S 903 was prepared according to a literature procedure (44). A sheet of S&S 903 filter paper is soaked in a saturated solution of DTPA for 24 hr, rinsed for 3 min in deionized water, and allowed to air-dry for 12 hr. The CM 23 resin on S&S 903 filter paper was prepared as follows. A weighted amount of carboxymethylcellulose resin (1.0 g) is hydrolyzed with 0.5 M NaOH (15 mL) following the instructions of the manufacturer (Whatman Laboratory Products). A 2 mL portion of the basic resin suspension is filtered over S&S 903 filter paper (4.25 cm diameter). The resin is then washed with deionized water, until the pH of the filtrate is approximately 10, and dried at room

temperature. Disks of 0.25 in diameter are cut with a standard paper punch and used as support for RTP.

Results and Discussion

Phosphorescence spectral characteristics. The indole compounds phosphoresce at room temperature and on filter paper when in the presence of a heavy atom enhancer. Only the RTP of 5-nitroindole was not observed under any of the experimental conditions used. The spectral characteristics of the indoles as measured on the Aminco-Bowman spectrofluorometer are very similar to each other (they are described in chapter 2). However, the presence of Tl(I) ions resulted in significant red shifts of the emission phosphorescence maxima and in a reduced vibrational structure, compared to the RTP spectra with I^-. Table 6 contains the maxima of excitation and emission for the indoles under the different experimental conditions.

Comparison of the RTP intensity on the different supports. Whatman DE-81, DTPA S&S 903, and CM 23 resin on S&S 903 were evaluated for their influence on the RTP relative intensities of several indoles, in the presence of I^- or Tl(I). These heavy atoms were used because in their absence, the RTP signal of the compounds was very low or undistinguishable from the background phosphorescence. Table 6 contains the relative RTP intensities of the indoles on the different substrates.

In the presence of I^-, DE-81 enhances to a variable extent the analyte intensities of all indoles compared to DTPA S&S 903 and CM 23 resin. The enhancement factors for DE-81 compared to DTPA S&S 903 ranged from 1.4 for indole to 3.0 for 2-methylindole. The enhancement

Table 6. Comparison of the Analytical RTP Characteristics of Indoles on Several Filter Papers and in the Presence of Heavy Atoms.

Compound[a]	Heavy Atom	Substrate[b]	λ_{ex}	λ_{em}	Relative Net RTP[c]	I_I/I_{Tl}[d]
Indole	I^-	DE-81	270	430	1.00	9.4
		DTPA	270	430	0.69	5.4
	Tl(I)	DE-81	280	465	0.11	
		DTPA	280	465	0.13	
3-Carbinolindole	I^-	DE-81	278	440	1.03	25
		DTPA	278	440	0.45	12
	Tl(I)	DE-81	278	466	0.04	
		DTPA	278	466	0.04	
5-Cyanoindole	I^-	DE-81	280	440	2.42	6.7
		DTPA	280	440	1.74	2.2
	Tl(I)	DE-81	280	445	0.40	
		DTPA	280	445	0.79	
5-Fluoroindole	I^-	DE-81	270	434	1.37	8.1
		DTPA	270	434	0.81	
		CM 23	270	434	0.94	0.9
	Tl(I)	DE-81	270	454	0.17	
		CM 23	270	440	1.06	
5-Methoxyindole	I^-	DE-81	278	437	0.65	9.0
		DTPA	278	437	0.27	3.2
	Tl(I)	DE-81	278	452	0.07	
		DTPA	278	452	0.08	
2-Methylindole	I^-	DE-81	269	428	0.67	14
		DTPA	269	428	0.22	6.4
		CM 23	269	428	0.24	1.5
	Tl(I)	DE-81	269	464	0.05	
		DTPA	269	476	0.03	
		CM 23	269	433	0.16	

[a] Indole concentration is 10^{-3} M.
[b] DTPA means DTPA-treated S&S 903 filter paper; CM 23 is the carboxymethylcellulose resin on S&S 903 filter paper.
[c] Net relative intensity was corrected for background phosphorescence intensity and normalized to the RTP of Indole (10^{-3} M) of 207.
[d] The ratio of the phosphorescence signal intensity with I^- to signal intensity obtained in the presence of Tl(I), using the same substrate.

observed for DE-81 when compared with CM 23 was 1.5 and 2.8 for 5-fluoroindole and 2-methylindole, respectively.

The fact that larger RTP signals of indoles are obtained on DE-81 than on the other papers may be attributed to the formation of hydrogen bonds which hold the analyte molecules more rigidly to the free hydroxyl groups that are present in the anion exchange filter paper DE-81, but not in the other papers. The anionic exchange capabilities of the filter paper may also contribute to the observed enhancement. The free hydroxyl ions in the surface may be exchanged against the iodide ions spotted on the surface. A better and stronger link could then be formed between support, heavy atom and phosphor. This type of interaction could not be observed on CM 23 which is a cationic exchange resin. The signals observed with CM 23, although slightly higher than those on S&S 903, were much lower than on DE-81.

In contrast, in the presence of Tl(I), the intensities of the indoles in DE-81 were approximately the same or slightly lower than on S&S 903. The use of Tl(I) on CM 23 gives larger signals for the compounds tested (5-fluoroindole and 2-methylindole). In the case of 5-fluoroindole, the CM resin enhances the signal about six times compared to DE-81 when Tl(I) is used as enhancer of RTP. This behavior may be due to the perturbation of hydrogen bonding in DE-81 by Tl(I) ions, which would result in a decrease of the RTP signal on this support. The adsorption of the planar 5-fluoroindole molecule at the surface of the CM resin may be stabilized by the interaction of

thallium ions with both the carboxymethylcellulose groups of this resin and the phosphor.

Effects of heavy atoms on the RTP intensities. The external heavy atom effects of I^- and Tl(I) on the RTP intensity of indoles were investigated on DE-81, DTPA S&S 903, and CM 23. Of the two heavy ions, I^- seems a much better enhancer of the RTP signals than Tl(I), as shown by the enhancement factors, I_I/I_{Tl} [the ratio of the RTP intensity of the indole in the presence of I^- to the intensity in the presence of Tl(I)], which ranged, depending on compound and support, from 2 to 25 except for 5-fluoroindole whose enhancement factor is 0.9. These results are in agreement with previous data showing a considerable enhancement of the RTP signals of unsubstituted indole and of tryptophan in the presence of sodium iodide (12,13). The favorable interaction of the Tl(I) ions with the cation exchanger is a probable cause for the exception observed for the 5-fluoroindole in this case.

Effect of substituent on RTP of indoles. As mentioned above, the position and the type of substituent do not change significantly the wavelength of the phosphorescence emission bands. In contrast, the relative RTP intensity varies with the type of substituent, between 2.42 for 5-cyano and 0.65 for 5-methoxyindole. Considering only a substituent in the same 5-position, the trend in relative intensities is 5-CN > 5-F > H > 5-MeO. This trend seems to follow approximately the order of the electron withdrawal interactions of the substituent. An increase in the polarity of the molecules, such as in 5-cyanoindole and 5-fluoroindole, should strengthen the hydrogen

bonding between these molecules and the active sites on the papers. These results may also support the hypothesis of a charge-transfer complex (2,4) between the indoles and iodide, with iodide acting as an electron donor. Other factors such as steric effects which can increase (or decrease) the planarity of the molecules, and therefore their rigidity, should also play a role in the RTP intensities of the indoles. For example, a cyano group is expected to increase the planarity and the rigidity of the indole molecule, which is in agreement with the high RTP intensity observed for 5-cyanoindole.

Study of pH and Substrate Effects on the RTP of Some Indolecarboxylic Acids

Introduction

The pH effect on the RTP of several indolecarboxylic acids was evaluated. Changes in pH determine the nature of many species and the ways in which they can interact with the solid substrate on which they are deposited. Therefore, pH-related studies are very important in the understanding of the type of interactions responsible for the occurrence of RTP. In addition, the possible mechanism of interaction of indolecarboxylic acids with different filter papers (DE-81, DTPA S&S 903, CM 23 on S&S 903 filter paper) at different pH have been investigated.

Experimental

Apparatus. All RTP spectra and intensity measurements were performed with an Aminco-Bowman spectrofluorometer. The description of the instrumental parameters are given in chapter 2.

Procedures. A 2 μL portion of sample (or solvent) was deposited onto the surface of the substrate, together with 2 μL (for DE-81) or

3 μL (for DPTA S&S 903 and CM 23) aliquots of 1 M KI. The measurements were done as described earlier (see chapter 2).

The indolecarboxylic acids studied were 3-indoleacetic acid, 3-indolebutyric acid, 3-indolelactic acid, 3-indoleacrylic acid, 3-indolepyruvic acid, and 3-indoxyl acetate. The solutions of the indolecarboxylic acids were prepared in neutral, acidic, and basic solvents. Neutral solutions were made up with an ethanol:water (50:50 v/v) mixture; acidic solutions were made approximately 0.1 M in HCl (pH ≈ 1.6); basic solutions were made approximately 0.5 M in NaOH (pH ≈ 13). Table 1 contains a list of the suppliers for all the reagents used in this study.

Results and Discussion

RTP spectral properties of the indolecarboxylic acids. All the indolecarboxylic acids under study showed RTP spectra, when adsorbed on DE-81 filter paper in the presence of 2 μL of 1 M KI, with the exception of 3-indoleacrylic acid, which gave no detectable phosphorescence signal from 2 μL of 10^{-3} M solutions. The RTP excitation and emission wavelength maxima are given in Table 7 for neutral, basic and acidic conditions.

It can be seen from Table 7 that the excitation and emission maxima do not change markedly with pH with the exception of a significant blue-shift on-going from pH 7 to pH 13 for indoxyl acetate. However, this latter change in the phosphorescence spectrum is probably due to hydrolysis in basic solution; indeed a rapid color change of the basic indoxyl acetate solution, from light yellow to violet occurs upon agitation. The absence of pH-related shifts of the

Table 7. Room Temperature Phosphorescence Properties of Indolecarboxylic Acids.

Compound[a]	pH Conditions	λ_{ex}[b]	λ_{em}[b]	Heavy Atom Enhancement[c]
3-Indoleacetic acid	Neutral	286	450	152
	0.5 M NaOH	288	450	
3-Indolebutyric acid	Neutral	287	448	300
	0.5 M NaOH	286	450	
DL-3-Indolelactic acid	Neutral	288	448	480
3-Indolepyruvic acid	Neutral	283	442	260
	0.5 M NaOH	286	442	
3-Indoxyl acetate	Neutral	266	466	
	0.5 M NaOH	323	434	4

[a] Concentration 10^{-3} M in ethanol:water (50:50 v/v) (2 µL). Samples were deposited on DE-81 filter paper.
[b] Precision of wavelength value ± 2 nm.
[c] Heavy atom enhancement factor defined as the ratio of analyte RTP net relative intensity in the presence of 1 M KI (2 µL) to analyte RTP net relative intensity without heavy atom.

emission spectra of most of the indolecarboxylic acids is in agreement with the results of Aaron et al. (84), who found that the low-temperature phosphorescence (LTP) spectra of these compounds did not change significantly with pH. It is apparent that RTP and LTP emission bands occur at very similar wavelengths (84), although the RTP bands are broadened and show little vibrational fine structure compared to LTP spectra, as previously observed for several other compounds (85). However, our RTP results, obtained on filter paper, are in contrast to those of Hurtubise and Smith (26), who recently found that when adsorbed on aluminum-backed silica gel chromatoplates, 2-indolebutyric acid and 1-methylindole-2-carboxylic acid did not show any RTP signal, although 5-indolecarboxylic acid did. This difference in behavior of the first two of these compounds, with the nature of the solid substrate used, seems to indicate that in the case of indolecarboxylic acids anion-exchange filter paper induces stronger phosphorescence signals than does silica gel.

Substrate and pH effects on RTP intensity of the indolecarboxylic acids. The influence of pH and substrate on the RTP intensity of indolecarboxylic acids was investigated, with neutral, basic (0.5 M NaOH) and acid (0.1 M HCl) solutions adsorbed on DE-81, DTPA S&S 903, and CM 23 resin on S&S 903 filter paper. In Table 8, the net RTP relative intensities for the indolecarboxylic acids deposited on the different substrates at various pH values are tabulated.

At acidic and neutral pHs the RTP intensities of most of the compounds tested were higher on DE-81 filter paper than on the DTPA and CM 23 supports. In basic solutions, the RTP signals on DE-81 and

Table 8. Comparison of RTP Intensities of Indolecarboxylic Acids on Various Substrates Under Various Conditions.

Compound[a]	Substrate[b]	pH Conditions[c]	Relative Net RTP[d]	$\frac{I_{Neutral}}{I_{pH}}$
3-Indoleacetic acid	DE-81	Neutral	50	
		Basic	31	1.6
	DTPA	Neutral	6.9	
		Basic	30	0.2
	CM 23	Neutral	9.1	
		Basic	12	0.8
3-Indolebutyric acid	DE-81	Acidic	15	1.7
		Neutral	26	
		Basic	22	1.2
	DTPA	Acidic	6.0	1.2
		Neutral	7.0	
		Basic	24	0.3
	CM 23	Neutral	9.6	
		Basic	5.4	1.8
DL-3-Indolelactic acid	DE-81	Acidic	11	7.2
		Neutral	79	
		Basic	19	4.2
	DTPA	Acidic	2.0	15
		Neutral	30	
		Basic	15	2.0
	CM 23	Acidic	3.7	
		Basic	7.0	
3-Indolepyruvic acid	DE-81	Neutral	1.0	
		Basic	2.4	0.4
	DTPA	Neutral	0.3	
		Basic	2.1	0.1
3-Indoxyl acetate	DE-81	Neutral	<0.2	
		Basic	22	<.01

[a] Compound concentration was 10^{-3} M.
[b] The substrate DTPA stands for DTPA-treated S&S 903 filter paper; CM 23 is the carboxymethylcellulose resin on S&S 903 filter paper.
[c] See text for preparation of solutions at different pH.
[d] The RTP intensity was corrected for background phosphorescence and normalized to the intensity of 2 µL of 10^{-3} M 3-indolepyruvic acid on DE-81; RSD = 10%.
[e] $I_{Neutral}/I_{pH}$ represents the RTP relative intensity of neutral solution compared to intensity of basic or acidic solution, with the same substrate.

DTPA are comparable and larger than on CM 23. In general, the highest signals were obtained from neutral solutions of the acids on DE-81. This paper also gave the largest signal for the neutral solutions of the substituted indoles, as mentioned previously. In contrast, basic solutions yielded a stronger signal on DTPA S&S 903; alkaline media enhanced the signal for the indolecarboxylic acids on DTPA S&S 903 by a factor of 2-6 relative to that of the neutral and acid solutions, except for 3-indolelactic acid where no enhancement was observed. The RTP intensity of all these compounds on CM 23 was relatively low, both in basic and neutral media.

The effect of pH on the RTP signals of indolecarboxylic acids suggests that various interactions occur between particular indole acid species and the substrates. Since the excited triplet-state pK_as of the indole acids range between 4.5 and 6.3 (84), only undissociated species can be present in solutions at pH 1.6. In aqueous ethanol solutions, there will be a mixture of the undissociated and the dissociated species, as shown by the pH of 4.5 for the aqueous solution of 2-indolecarboxylic acid, for which pK_a is 4.9 (84). Finally, only the indolecarboxylate species will be present in solutions at pH 13.

On DE-81 filter paper, the mixture of dissociated and undissociated forms of the acids present in neutral solutions should remain on the dry surface and form various hydrogen bonds with the anion-exchange OH^- groups as well as with the neutral cellulose OH groups of the paper. Neutralization reactions which can occur at the wet surface between the undissociated species of the acids and the

free hydroxyl groups can also play an important role in determining the type of interactions prevailing in the dry stage. Acid-base reactions are less probable on DTPA S&S 903 or CM 23. Hydrogen bonding seems to be the main mechanism of interaction under these conditions. When basic test solutions are used, electrostatic repulsion between the carboxylate groups and the OH^- groups of the DE-81 paper will diminish the overall hydrogen bonding, thus reducing the RTP intensity.

It is surprising that the phosphorescence signals from DTPA S&S 903 paper were higher for alkaline solutions (pH 13), than for neutral or acidic solutions. In the high-pH test, DTPA should initially react with the sodium hydroxide, and on drying exist only as the pentasodium salt, since pK_as is 10.56 (86). Electrostatic repulsion between the indolecarboxylate and DTPA acetate groups should then prevent formation of hydrogen bonds with the hydroxyl groups of the paper. However, since the sodium hydroxide content of the 2 μL sample is only 0.1 μmole, although the pH is > 13, the composition of the dried spot will depend on the loading of DTPA on the paper and the extent of diffusion of the sample spot before drying. The indolecarboxylate anions seem to interact well with the hydroxyl groups of the cellulose paper. It is not clear whether the increase of the RTP signals of indolecarboxylates when spotted from alkaline solutions onto DTPA S&S 903 is caused by hydrogen bonding or by packing of the DTPA sodium salt into the filter paper. The latter could cause inhibition of the internal molecular motions of the phosphor, analogously to the

interaction mechanism proposed by Niday and Seybold (5) to explain the effect on RTP of several salts and sugars packed into the paper.

Heavy-atom effect on RTP intensity. The "heavy atom" effect of iodide on the RTP response curves was studied for two different concentrations of 3-indolebutyric acid on DE-81 substrate. The RTP response curves are shown in Fig. 6. They are linear over a range of about one order of magnitude. The curves decrease for iodide concentrations > 2 M. Similar iodide response curves were observed by Su and Winefordner (34) for several drugs and pesticides deposited on DE-81.

Table 7 presents the heavy-atom enhancement factors for several indole acids. With the exception of 3-indoxyl acetate, the RTP signals were extremely weak in the absence of a heavy atom, but relatively strong when 2 µL of 1 M KI were used. Therefore, heavy-atom enhancement factors are generally very large, ranging between 4 and 480 depending on compound.

Substrate, Heavy Atom, and pH Effect on the RTP of Diazepam

Introduction

Diazepam is a very good example to illustrate the selectivity inherent to RTP. It absorbs strongly in the near UV region (242, 285 nm) (76), does not fluoresce at room temperature, but phosphoresces when spotted on filter paper in the presence of mercuric chloride. The observation of the environmental effect on the RTP properties of diazepam not only helps to define the optimum experimental conditions for its determination in mixtures (e.g., in pharmaceutical formulation

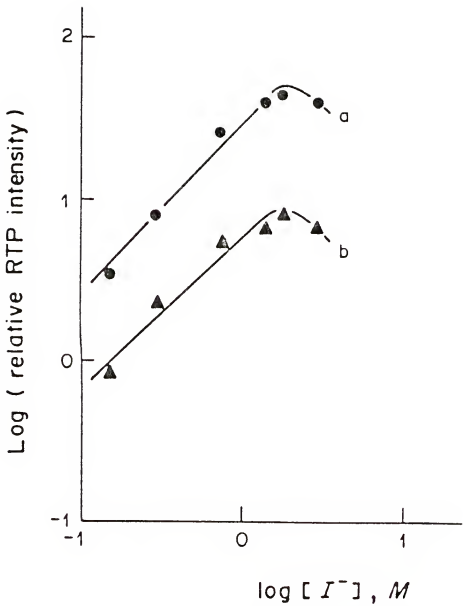


Fig. 6. Heavy-atom RTP response curves for 3-indolebutyric acid on DE-81 filter paper, in neutral water:ethanol (50:50 v/v) solutions. Concentrations of 3-indolebutyric acid: 10^{-3} M (curve a), 10^{-4} M (curve b).

and in serum), but also provides information which will help us to understand better the interactions at the surface of the support.

Pure cellulose as well as cation and anion exchange filter papers are evaluated as substrates for the RTP of diazepam. Whatman No. 1 is a pure cellulose filter paper (content > 99% cellulose, according to manufacturer specifications). The cationic exchange filter paper Whatman P-81 has free Na^+ ions as well as dibasic phosphate groups bonded to the cellulose polymer, while the anionic exchange filter paper Whatman DE-81 is a diethylaminoethyl cellulose polymer containing free OH^- groups. Filter paper DE-81, as mentioned before, is a very good substrate for RTP of neutral solutions of the indoles in the presence of KI.

The heavy atom effect on the RTP of diazepam is investigated. Compounds of Tl(I), Ag (I), Pb(II), Hg(II), and I^- are evaluated as heavy atom enhancers.

The pH effect on the phosphorescence intensities of diazepam is also expected to be important, since organic compounds containing ionizable sites are known to exhibit stronger phosphorescence when spotted from acidic solutions (26,81,83). In this study, the RTP of diazepam spotted from neutral (pH ≃ 6.2) and acidic (pH ≃ 1.6) solutions are compared.

Experimental

All RTP spectra and intensity measurements were performed with the Perkin-Elmer Model LS-5 fluorescence spectrometer. The instrumental parameters and measurement process for collection of RTP data from diazepam are described in chapter 2.

Neutral solutions of diazepam were made up with ethanol. Acidic solutions were prepared with 0.1 M HCl in ethanol:water (50:50 v/v). The heavy atom compounds used were $HgCl_2$, KI, $Pb(C_2O_2H_3)_2$, $TlNO_3$, and $AgNO_3$. The supports were Whatman No. 1, DE-81, and P-81. The suppliers for all the reagents and materials used can be found in Table 1.

Results and Discussion

RTP spectral properties. Diazepam phosphoresces most intensely at room temperature in the presence of $HgCl_2$ as compared to the other heavy atom enhancers. The RTP emission and excitation spectra can be seen in Fig. 5. There are slight shifts in the spectrum of diazepam when changing the microenvironment. The excitation and emission maxima obtained from ethanolic solutions of diazepam deposited on pure cellulose filter paper Whatman No. 1 and the cationic and anionic papers Whatman P-31 and DE-81, respectively, and in the presence of Ag(I) and Hg(II), are given in Table 9. No phosphorescence was observed from diazepam spotted on Whatman DE-31 under neutral conditions. Of the heavy atoms tested as enhancers for RTP of diazepam, phosphorescence was only observed with Hg(II) and Ag(I), and with the latter only on P-81.

Substrate and heavy atom effects. The relative phosphorescence intensity of diazepam depends on the paper substrate, the heavy atom present on the support, and the hydronium ion concentration of the wet paper surface (at the moment of spotting). Strong phosphorescence signals are obtained when the filter papers Whatman No. 1, P-81, and DE-81, are used as supports in the presence of an acidic solution of

Table 9. Diazepam RTP Properties Using Several Filter Papers and Heavy Atoms Under Neutral Conditions.

Support	Heavy Atom[a]	λ_{ex}[b]	λ_{em}	RTP Relative Intensity[c]	Relative Background Phosphorescence[d]	I_D/I_B[e]
Whatman No. 1	Hg(II)	306,389	469	1.0	1.0	3.6
P-81	Hg(II)	305,386	466	2.5	3.8	2.3
	Ag(I)	297,306, 386	466	0.75	5.6	0.5
DE-81[f]	Hg(II)	306,389	469			

a Concentration of $HgCl_2$ is 0.1 M in water:ethanol (50:50 v/v). Concentration of $AgNO_3$ is 0.1 M in water.

b Wavelength of main peak is underlined. Precision of the wavelength values ± 2 nm.

c Relative net RTP intensity was corrected for background phosphorescence intensity and normalized to the RTP intensity (32) of diazepam (10^{-3} M) on Whatman No. 1. RSD = 10%.

d Relative background phosphorescence intensity of the paper with heavy atom solution was measured at $\lambda_{ex}/\lambda_{em}$ maxima of diazepam. It was normalized with respect to the background phosphorescence (9) of Whatman No. 1 with $HgCl_2$. RSD = 2%.

e Ratio of net diazepam phosphorescence intensity, I_D, to background phosphorescence, I_B.

f Only observed in an acidic environment.

$HgCl_2$. Weak emission is observed with Whatman No. 1--Hg(II) and with P-81--Ag(I) support-heavy atom combinations in a neutral environment. This is interesting in view of the large enhancement factors obtained for the indole derivatives in the presence of iodide. The mechanisms of interaction between a heavy atom and the phosphor which give rise to the heavy atom effect are not clearly understood (4,16,17,34). Table 9 shows the relative intensities of the RTP of diazepam under neutral conditions and in the presence of $HgCl_2$ and $AgNO_3$.

The higher intensity obtained with P-81 compared to Whatman No. 1 and DE-81 (none observed from the latter in a neutral medium) indicates that interactions between analyte-heavy atom-substrate at the surface of P-81 are efficient in minimizing the nonradiative relaxation processes. The phosphate group ester-linked to the cellulose matrix of the paper can chemically interact with Hg(II) deposited on the surface. A variety of oxo-mercuric complexes containing -Hg-O- bonds are known (87). Mercury can also form a variety of complexes with nitrogen-containing organic compounds. In fact, the affinity of Hg(II) for nitrogen ligands in aqueous solution exceeds that of the other transition metals (87). The possible interactions of mercury with both the analyte molecule and the phosphate functional groups of the paper could give rise to a sandwich type complex. A link between the analyte and the support develops which together with a heavy atom effect provides the necessary rigid environment. This is similar to the mechanism proposed by Lue-Yen Bower and Winefordner (17) with the heavy atom forming a complex with

aromatic compounds through interaction with the π electron system of the molecule while interacting also with the free hydroxyl groups on the paper. A similar mechanism was proposed earlier as a probable reason for the enhancement observed in the RTP of 5-fluoroindole spotted on CM 23 in the presence of Tl(I).

Whatman No. 1 is a pure cellulose paper with no functional groups capable of interacting strongly with the heavy atom or the analyte. With no possible hydrogen bonding, only weak dispersive forces (4) help to maintain a rigid matrix. This explains the lower intensities of the phosphorescence signal of diazepam spotted on Whatman No. 1. DE-81 contains diethylaminoethyl functional groups. The positively charged tertiary amine cannot interact with the Hg(II) and may, in fact, prevent diazepam from being rigidly held on the surface. No phosphorescence is observed under these conditions.

Interactions of pH

The hydronium ion concentration of the wet surface of the paper has a marked influence on the RTP intensity of diazepam. The analyte solution or the heavy atom solution was made acidic with 0.1 M HCl, and the effect on the RTP signal was compared to the signal obtained under neutral conditions. The enhancement observed in the intensities depended on the type of filter paper used as substrate. In Table 10, the net relative intensities are given for acidic and neutral conditions for the different substrates studied. Adding 2 or 3 μL of 0.1 M HCl solution to the paper prior to the spotting with neutral heavy atom and analyte solutions has an effect similar to using an acidic heavy atom solution. The excess hydronium ion concentration

Table 10. Comparison of the RTP Relative Intensities of Diazepam in Neutral and Acidic Environments Using Different Substrates.

Support	pH Conditions[a]	RTP Net[b,c] Relative Intensity	$\frac{I_{acid}}{I_{neutral}}$ [d]
Whatman No. 1	Neutral	1.0	
	Acidic	133.0	133.0
P-81	Neutral	2.5	
	Acidic	69.0	27.0
DE-81	Neutral	< 0.19	
	Acidic	37.0	> 195.0

[a] Neutral and acidic conditions were produced by using neutral ethanol:water (50:50, v/v) and acidic (0.1 M HCl in ethanol:water, 50:50, v/v) solutions of $HgCl_2$.

[b] Diazepam concentration was $1.2X10^{-3}$ M and $8.8X10^{-5}$ M for measurements under neutral and acidic conditions, respectively. $HgCl_2$ concentration was 0.1 M.

[c] Room temperature phosphorescence net relative intensity was corrected for background phosphorescence intensity and normalized to the RTP intensity of $1.2X10^{-3}$ M Diazepam (32) on Whatman No. 1 in a neutral environment.

[d] $I_{acid}/I_{neutral}$ represents the RTP relative intensity of the acidic compared to the neutral conditions using the same substrate.

present on the wet surface at the moment of spotting diazepam is probably sufficient to protonate the molecule producing the conjugate acid of diazepam whose pK_a has been reported (88) to be 3.4. Diazepam in its acid form is able to hydrogen bond to the hydroxyl groups of the support. This explains the significant increase of the phosphorescence signal as compared to neutral conditions.

The enhancement is greater for DE-81 (> 195 when RTP intensity in acidic conditions is ratioed to the LOD under neutral conditions). The molecule is now capable of hydrogen bonding to the free OH^- groups present in DE-81 (although partial neutralization of these groups is expected due to the acidic environment), as well as to the hydroxyl groups of the cellulose matrix. The latter are also responsible for the enhancement observed on Whatman No. 1.

Hydrogen bonding is probably also present on P-81. The excess of hydronium ions in solution could partially neutralize the negative charges of the phosphate groups linked to the cellulose matrix. This would disrupt the type of interaction present at the surface under neutral conditions, specifically the "sandwich type complex" proposed earlier. There may also be some electrostatic repulsion between the positively charged phosphor and the acidic surface. The signal obtained from P-81 in an acid environment is enhanced to a lesser degree as compared to DE-81 and Whatman No. 1.

Under acidic conditions Whatman No. 1 becomes a better substrate. Since the phosphorescence signal is higher on Whatman No. 1, and the background phosphorescence of the filter paper is low (see Table 9), Whatman No. 1 is a better support for the RTP of

diazepam. It was, therefore, selected as support for the RTP quantitative determination of diazepam in its pharmaceutical formulation and in serum (see chapter 2).

Hydrogen bonding is an important mechanism of interaction for the protonated diazepam molecule but cannot be used to explain the results observed under neutral conditions. Heavy atom-support interactions seem to play an important role in the RTP of neutral solutions of diazepam.

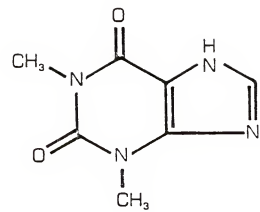
Substrate and pH Effects on the RTP of Caffeine and Theophylline

Introduction

Caffeine, a stimulant, and theophylline, a bronchodilator, are two methylxanthines of considerable importance, both for the scientific and lay communities. Few reports have been published on the luminescence properties of theophylline (48,89) and even fewer on those of caffeine (47,90). The RTP properties of these compounds have been observed by Bateh and Winefordner (47,48) who made use of the greater spectral selectivity of RTP to develop a simple and specific assay for caffeine and theophylline in pharmaceutical formulations. The phosphorescence of caffeine and theophylline was observed from neutral solutions of the compounds deposited on DTPA-treated S&S 903, and in the presence of KI (47,48).

In view of the pH and substrate effects observed in the RTP of the indole derivatives and diazepam, it is of interest to observe these effects on the RTP of caffeine and theophylline, nonaromatic nitrogen heterocyclics containing several functional groups (see Fig. 7). The enhancement observed in the phosphorescence of the indole

a.



b.

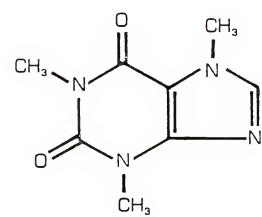


Fig. 7. Molecular structures of theophylline (a) and of caffeine (b).

derivatives when filter paper DE-81 is used in place of DTPA S&S 903, and in the phosphorescence of diazepam when the compound is spotted from an acidic solution suggested that optimum experimental conditions for the analysis of caffeine and theophylline could be found from a study of the substrate and pH effects on the RTP of these compounds. This could provide greater sensitivity and selectivity which would be beneficial in the application of RTP to the analysis of the methylxanthines in "real life samples."

The cellulose filter paper Whatman No. 1, and the ionic exchange papers P-81 and DE-81 were used as supports. The signals observed from acid, neutral, and basic solutions of caffeine and theophylline deposited on different filter papers were compared.

Experimental

Apparatus. The excitation and emission RTP spectra were obtained with the LS-5 fluorescence spectrophotometer, interfaced to a model 3600 data station. The instrumental parameters were similar to those used in the diazepam studies.

Materials. Caffeine and theophylline were used as received. $HgCl_2$, $TlNO_3$, KI, $PbNO_3$, and $AgNO_3$ were tested as enhancers of the RTP of the methylxanthines. Absolute ethanol, nanopure deionized water, HCl, and NaOH were used in the preparation of the solutions. Whatman No. 1, DE-81, and P-81 were used as supports for the RTP studies. A list of the suppliers/manufacturers can be seen in Table 1.

Procedure. Neutral solutions containing 10 µg/mL of caffeine and theophylline were prepared in ethanol:water mixture (50:50 v/v).

Acidic solutions were made approximately 0.1 M HCl and basic solutions were made approximately 0.1 M NaOH.

Procedures for the measurements were the same as those used for the diazepam studies (see chapter 2).

Results and Discussion

Heavy atom effect. Neither caffeine nor theophylline phosphoresce in the presence of positively charged heavy atom ions such as Tl(I), Ag(I), Hg(II), or Pb(II). In contrast, I^- enabled the observation of the RTP of the methylxanthines. It is interesting to notice that although theophylline is known to form stable complexes with Ag(I) (91), and weak interactions are expected for the external heavy atom effect (4), no phosphorescence could be detected from either compound in the presence of Ag(I).

The response curve (relative RTP intensity vs KI concentration) for a 10 μg/mL neutral solution of caffeine deposited on DE-81 can be seen in Fig. 8. The optimum KI concentration for maximum RTP intensity is approximately 2 M. Similar results were observed previously for the RTP of 3-indolebutyric acid on DE-81 in the presence of iodide.

Substrate and pH effects. Neutral, acidic (0.1 M HCl), and basic (0.1 M NaOH) solutions of caffeine and theophylline were spotted on the three different types of filter paper. The excitation and emission spectrum of caffeine and theophylline are very similar to each other. The RTP spectrum of neutral solutions of the compounds spotted on Whatman No. 1 and in the presence of 1 M KI can be seen

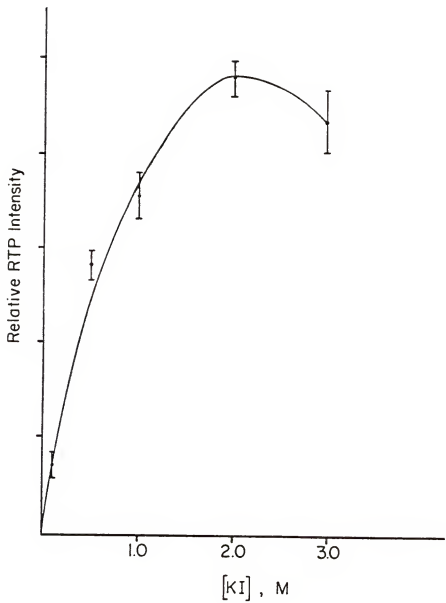


Fig. 8. Iodide RTP response curve for 10 μg/mL neutral water:ethanol (50:50 v/v) solution of caffeine on DE-81. Aliquots of 3 μL of KI and of caffeine solutions were used.

in Fig. 9. The maximum of the excitation and emission occurred at 282 and 445 nm, respectively. The broad emission band lacks structural features and is characteristic of phosphors with and without the presence of heavy atoms. Two shoulders around 258 and 273 nm can be observed in the excitation spectrum.

The RTP spectrum did not vary appreciably with the type of support or the pH of the spotting solution. Slight variations occurring from experiment to experiment are probably due to variations in the background phosphorescence, which cannot be compensated for. It seems that the interactions between the support, the heavy atom and the phosphor are similar for both compounds as expected, since, structurally, they are very similar.

Although the spectral features are not apparently influenced by the microenvironment of the phosphor, the intensities of the signal are affected; Fig. 10 shows the relative intensities of caffeine and theophylline under different experimental conditions. The compounds have different responses to different environments. Theophylline, however, seems to be more sensitive than caffeine to the microenvironment. The intensity of neutral solutions of theophylline in DE-81 is 14% of its value on Whatman No. 1. The basic properties of the anion exchange filter paper DE-81 seems to affect to a greater extent the phosphorescence emission of theophylline. This is expected considering the pK_as (83) of caffeine (pK_a = 14) and of theophylline (pK_a = 8.6). Similar observations can be made on the LTP of the compounds in basic matrices (92). It is comparatively easier to form

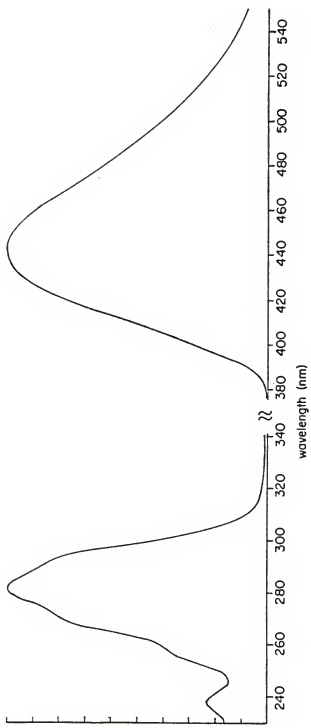


Fig. 9. Excitation and emission RTP spectrum of 10 μg/mL theophylline (caffeine spectrum is similar) on Whatman No. 1 and in the presence of 1 M KI.

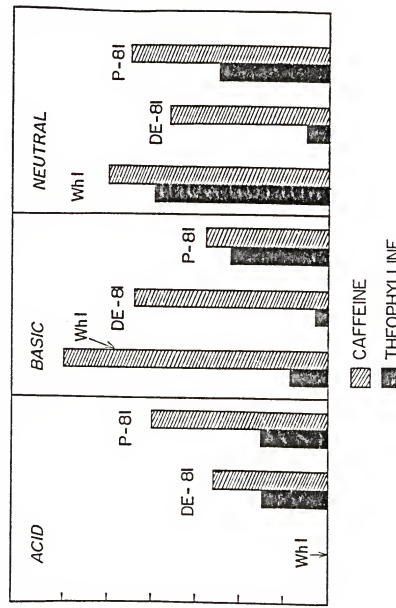


Fig. 10. Relative RTP intensities for 10 ug/mL of caffeine and of theophylline on different supports and from different solvent systems; 3 uL of 1 M KI added.

the anion of theophylline whose quantum yields seem lower than that of the neutral species, as observed from LTP studies (92). Similar results are obtained when basic solutions of theophylline are spotted on Whatman No. 1. The basicity of the solvent or of the substrate results in a considerable reduction on the emission of theophylline relative to the neutral medium. The response of caffeine to basic media is not as marked and as dependent on the type of support. The highest caffeine intensities are observed from basic solutions spotted on Whatman No. 1.

No phosphorescence above background is observed from acid solutions of caffeine and theophylline spotted on Whatman No. 1. There may be a poor interaction between the substrate and analyte due to protonation of the organic molecule and/or protonation of the active sites of the cellulose polymer, causing a considerable reduction in the emission intensities. It is also possible for the phosphorescence quantum yields of the protonated species to be very low. A considerable reduction in the phosphorescence intensity was also observed for acidic matrices at low temperatures (92). However, phosphorescence is observed from acid solutions of caffeine and theophylline when these are spotted on DE-81 or P-81. The presence of the free hydroxyl groups in DE-81 or the phosphate groups attached to the cellulose polymer of P-81 may help to neutralize or counteract the acidity effects of the solvent. Acid-base neutralization reactions occurring during the initial "wet" stage of the support have been postulated by Ramasamy and Hurtubise (83). If a large portion of the

3 μL of 0.1 M HCl are neutralized by the active sites of the paper, then both neutral and cationic forms of the compounds could be present. It may also be possible that the cations of the methylxanthines are able to donate a proton to the hydroxyl or phosphate groups on the paper. Neutral species will then be present on the paper and phosphorescence will be observed.

In general, the response of the compounds on different supports to the acidity or basicity of the solvent varies. Whatman P-81 seems to be the least active and Whatman No. 1 the most. The mechanism of interaction present under various conditions is dependent on the molecular structure and on the microenvironment that surrounds it. A simple hydrogen bonding mechanism does not explain the observed behavior of the methylxanthines. Theophylline could hydrogen bond to the hydroxyl groups of the paper through the hydrogen of its nitrogen-7 position. Caffeine has no acidic hydrogen but its phosphorescence is more intense than that of theophylline under all experimental conditions studied. Acidic and basic solvents which are known to enhance the phosphorescence of many organic compounds with polar functional groups (4) have the opposite effect on the phosphorescence of theophylline (the same observation can be made for caffeine when spotted from acidic solvents). The specific interactions between the support and these molecules cannot at present be elucidated. The molecular structure and the chemistry occurring at the wet surface, at the moment of spotting, plays an important role in the observation of RTP.

CHAPTER 4
SURFACE ANALYSIS OF FILTER PAPERS THROUGH X-RAY PHOTOELECTRON SPECTROSCOPY

Introduction

Solid substrate room temperature phosphorescence is a surface process. Radiative emission is observed from molecules rigidly held at the surface of the support. Analysis of the surfaces from which the phosphorescence is observed could help us understand better the nature of the interactions and processes occurring at the surface of the support. X-ray photoelectron spectroscopy (XPS) was the technique selected to analyze and study the surface of the substrate with and without the presence of heavy atoms and/or luminescent molecules. Soft X-rays provide a nondestructive tool that is also surface sensitive. XPS provides information about the elemental composition and the chemical oxidation states of the surface with a sensitivity of parts per thousand (93,94). It allows the qualitative and semi-quantitative analysis of filter papers spotted with heavy atoms/or phosphor solutions, provided they are present in amounts within the limit of detection of the particular element used as the probe. Elements are identified through the characteristic binding energies of their photoelectrons. The relative amounts of heavy atoms and of molecules on the surface of the paper can be determined from the photoelectron peaks. The photoelectron peaks are normalized with respect to time, the photoelectron cross-section [Scofield X-ray

absorption cross-sections (93)] and the instrumental factor (which is dependent on the kinetic energy of the photoelectron peaks). A discussion and the mathematical expression used in the normalization of the photoelectrons peaks can be found in Appendix A. The extent of penetration of the analyte and of the heavy atom was observed through grazing angle experiments. Varying the angle of observation of electron exit with respect to the surface allows us to examine layers of different thicknesses (Fig. 11).

Pure cellulose filter paper Whatman No. 1 was selected for the surface studies. Cellulose supports are commonly used as substrates for RTP. A review on their evaluation as RTP substrate can be found in chapter 1.

There are several requirements that must be met by the lumiphors and heavy atoms to be used as probes. The compound must luminesce or specifically phosphoresce when spotted on filter paper. It must have a very low vapor pressure to withstand the high vacuum conditions under which the XPS analysis is performed. The compound solubility must be $> 10^{-3}$ M in water or ethanol to allow for sufficient surface coverage. In order to increase the sensitivity toward XPS, the presence of an element with a high photoionization cross-section is desired. Salts of the heavy atom enhancers, I^-, Tl(I), and Ag(I), and the luminescent probe molecules, bis(8-quinolinate)platinum(II), 3,5-diiodotyrosine, 5-hydroxytryptophan, and carbaryl were used as probes. In order to observe the effect of heavy atom-containing surfactant salts on the surface of the paper (see also Appendix B),

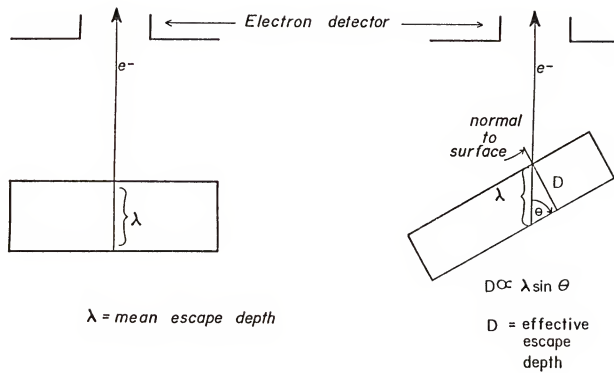


Fig. 11. Diagramatic presentation of the parameters influencing the effective escape depth (D).

salts of Tl(I) dodecyl sulfate (TlDS), and Ag(I) dodecyl sulfate (AgDS) were prepared, characterized, and used in the study.

The good heavy atom enhancers [I^-, Tl(I), and Ag(I)], which are commonly used in RTP, have relatively high cross-sections for X-ray absorption (94). Tyrosine phosphoresces at room temperature in the presence of KI (13). A substituted tyrosine containing two iodine atoms per molecule was chosen so as to enhance its sensitivity toward XPS. The iodine photoelectron peak is used to determine the relative amount of 3,5-diiodotyrosine present at the surface of the filter paper. Both 5-hydroxytryptophan and carbaryl are good phosphors (27,95). The phosphorescence emission intensity of these compounds is increased by the addition of heavy atoms such as I^- (for 5-hydroxytryptophan) and Tl(I) (for carbaryl).

The use of heavy-atom containing surfactants in place of inorganic salts is expected to increase the amount of heavy atoms available at the surface of the support. Filter papers treated with surfactant and inorganic salts were analyzed and the results compared. In addition, the phosphorescence properties of carbaryl spotted on Whatman No. 1 in the presence of both types of salts were observed. The results are correlated with those obtained from the surface analysis of filter papers treated with the Tl(I) and Ag(I) salts. The description of the experiments on the phosphorescence of carbaryl in the presence of the surfactant salts is given in Appendix B.

Experimental Section

Apparatus

The XPS studies were made with a Kratos XSAM 800 photoelectron spectrometer. The collection, presentation, and quantification of the data were done with a DS800 Data System. The spectra were obtained using nonmonochromatized Mg K_α radiation (1253.6 eV) with a base pressure of 10^{-8} Torr and typical operating parameters of 12.5 kV and 17 mA. The instrument is operated in the fixed retarding ratio mode. An electron flood gun (operated at 2 Å) was used at all times to minimize differential charging of the filter paper.

Reagents

Filter paper Whatman No. 1 was used as substrate. The chemicals KI, $TlNO_3$, $AgNO_3$, 5-hydroxytryptophan, and 3,5-diiodotyrosine were purchased (see Table 1) and used as received. Carbaryl was donated by the Environmental Protection Agency (USA).

A platinum salt, K_2PtCl_4, and the compound 8-quinolinol were used in the preparation of the platinum complex. Sodium dodecyl sulfate and the inorganic salts $AgNO_3$ and $TlNO_3$ were used for the preparation of the surfactant salts of Tl(I) and Ag(I).

Procedure

Synthesis of the platinum complex. The bis(8-quinolinate)-platinum(II) complex was synthesized as described in the literature (96). The 8-quinolinol compound was dissolved in a small volume of ethanol and added to an aqueous alkaline (pH 10) solution of K_2PtCl_4 in a mol ratio of 2:1. The resulting solution was heated on a steam bath for a few minutes. A brownish-orange precipitate appeared which

was filtered and recrystallized from N,N-dimethylformamide (DMF). The absorption spectrum showed the features characteristic of the 8-quinolinate platinum complex.

Preparation of surfactant salts. The dodecyl sulfate salts of thallium(I), TlDS, and of silver(I), AgDS, were prepared using a procedure similar to that by Humphry-Baker et al. (97).

Qualitatively the purity of the batches of TlDS and AgDS were verified by infrared spectrometry in the range of 4000 to 400 cm^{-1} and using the Nujol mull technique. The four intensity peaks in the region of strong characteristic absorption of nitrate (at 1382 and 823 cm^{-1}) and of NaDS (at 1243 and 1222 cm^{-1}) suggested a content near 90%. This was confirmed by Tl and Ag analysis using atomic absorption or emission techniques; contents of 88.7 and 86.5%, respectively, were found. The solubilities of the TlDS and AgDS in water at room temperature is less than 0.02 M. In order to examine the effect of these compounds at higher concentrations, ethanol and 2-propanol were used as solvents, since increased solubility of the surfactant salts (> 0.02 M) in these solvents were observed.

Measurements

Samples of filter paper Whatman No. 1 were taken from the middle of the box. A stack of papers were perforated to produce 1 cm diameter circles or disks. Disks from the middle of the stack were chosen since these have been less exposed to air and used immediately to avoid contamination. The disks were mounted on the spectrometer probe with double-sided tape. Both treated and untreated filter paper disks were analyzed. Papers were spotted after mounting with 6 μL of

the heavy atom and/or the luminescent compound solutions by means of an SMI micropipetter. The samples were placed in the spectrometer immediately to avoid prolonged exposure to the atmosphere; they were dried approximately 2 hr at room temperature and under vacuum (10^{-3} Torr) inside the treatment chamber of the spectrometer.

The following heavy atom solutions were used in the studies: 0.01, 0.5, 1.0, and 2.0 M KI aqueous solutions; 0.02 and 0.1 M $TlNO_3$ in water; 0.02 M TlDS in ethanol; 0.02 M $AgNO_3$ in water; and 0.02 M AgDS in isopropanol. The molecular probe solutions were 0.002 M 3,5-diiodotyrosine in ethanol, 0.01 M 5-hydroxytryptophan in water, 0.005 M carbaryl in ethanol, and a saturated solution of bis(8-quinolinate)platinum(II) complex in DMF.

Potassium iodide and 3,5-diiodotyrosine pellets were made with a pellet press. Both the die plug and the die pellet receiver of the press were thoroughly cleaned and dried before use. The pellets were mounted on the probe with double-sided tape and placed in the spectrometer for analysis.

Results and Discussion

XPS Studies of Whatman No. 1 Filter Paper

The XPS spectrum of Whatman No. 1 filter paper shows mainly C 1s and O 1s photoelectron peaks. The C 1s peak is observed to consist of three main classes of carbon atoms present in wood components (98). Class I carbon atoms are those bonded to only carbon or hydrogen atoms and class II and class III carbon atoms have one and two bonds with oxygen atom, respectively. The relative abundance of each class of carbon atoms on the surface was determined by computer curve fitting

of the different carbon peaks. The observed chemical shifts and fractional areas of these peaks are in reasonable agreement with previous reports (99). The results of the analysis of Whatman No. 1 are presented in Table 11.

The cellulose polymer contains only class II and III carbon peaks. Experimental results show that the highest contribution to the C 1s peak comes from these two classes of carbon atom as evidenced from their fractional areas (see Table 11). Class I carbon atoms, as observed from Table 11, constitutes 10% of the carbon atoms on this particular sample. The fractional areas of class I carbon atoms vary significantly from sample to sample. For example, C 1s peaks containing up to 25% contribution from class I carbon atoms have been obtained. Although this contribution may be due to residual impurities present on the paper, it seems that the major contribution comes from contamination from the atmosphere or the spectrometer vacuum system.

The average ratio of the normalized areas of oxygen to those of class II and III carbon atoms as determined from the analysis of 11 samples was 0.83 ± 0.05. The ratio compares favorably with the theoretical value (0.83) and the literature reported value (0.79 ± 0.04) (98).

Surface Analysis of Filter Paper Treated With Heavy Atoms and/or Lumiphor Solutions

The presence of compounds containing elements other than only carbon and oxygen can be readily detected on the surface of the filter paper provided the element sensitivity is high and/or the compound surface coverage is large. The binding energies of the observed

Table 11. X-ray Photoelectron Spectroscopy Data for Filter Paper Whatman No. 1.

Element	Class	Binding Energy (eV)[a]	Chemical Shift (eV)[b]	Fractional Areas[b]	$O/(C_{II}+C_{III})$
Carbon	I	-C-C (H)	285.0	--	9.7(7.6)	--
	II	-C-O	286.4(288.2)	1.4(1.7)	76.9(70.9)	--
	III	-C-O (O)	287.6(288.6)	2.6(3.2)	13.3(21.7)	--
Oxygen			532.8(533.5)[c]	--	--	0.83

[a] The binding energies are referenced to the 285 eV hydrocarbon C 1s peak.

[b] Values in parentheses were taken from reference 98. They are included for comparison purposes.

[c] Oxygen binding energy is taken from reference 99; it is the value corresponding to a McPherson ESCA 36 spectrometer.

photoelectron peaks are referenced to the class I (hydrocarbon-type) C 1s peak at 285.0 eV. The binding energies for the different photoelectron peaks observed from the surface analysis of the treated papers are tabulated in Table 12. The I $3d_{5/2}$ binding energies as observed from the analysis of KI and 3,5-diiodotyrosine pellets are also included.

The energy of an emitted core electron may be altered depending on the type of chemical bond formed by the element in question. There is a shift of 2 to 4 eV toward higher binding energy of the I $3d_{5/2}$ photoelectron peak in 3,5-diiodotyrosine as compared to that of ionic iodine in KI as observed from the measurement of pellets and of papers treated with these compounds. The differences in binding energies reflect the differences in the chemical state of iodine in these two compounds. There is no evidence of a strong interaction of I^- with the cellulose fiber.

Complex formation or strong chemical interaction between I^- or Tl(I) with the organic phosphor could alter the observed binding energies of the ions. A chemical shift of approximately 2 to 4 eV toward higher binding energies is expected for iodine based on the results obtained for ionic and covalently bound iodine atoms. As seen from the results in Table 12, the presence of 5-hydroxytryptophan and carbaryl do not alter significantly the binding energies of I^- or of Tl(I), respectively. There is no evidence from the XPS studies of strong chemical interaction between the heavy atom and the phosphor spotted on the surface of the paper. The shifts observed on the C 1s class II and class III peaks of the treated papers are very small

Table 12. Photoelectron Binding Energies From XPS Spectra of Treated Filter Papers (and of I $3d_{5/2}$ From Analysis of Pellets).

Spotting Solutions[a]	C 1s (II)[b]	C 1s (III)[c]	O 1s	I $3d_{5/2}$	Tl $4f_{7/2}$	Ag $3d_{5/2}$
1.0 M KI ((5))[d]	287.3 (0.2)[e]	289.1 (0.2)	533.9 (0.1)	619.7 (0.2) [619.9 (0.6)][f]	-	-
1.0 M KI and 0.01 M 5-hydroxytryptophan ((2))	286.7 (0.2)	288.4 (0.5)	533.4 (0.3)	619.3 (0.4)	-	-
$2X10^{-3}$ M 3,5-diiodotyrosine ((2))	286.7 (0.3)	288.4 (0.4)	533.7 (0.3)	622.1 (0.3) [623.6 (0.4)]	-	-
0.1 M $TlNO_3$ ((3))	287.3 (0.1)	289.0 (0.1)	534.0 (0.2)	-	120.8 (.6)	-
0.1 M $TlNO_3$ and $5X10^{-3}$ M Carbaryl ((2))	287.2 (0.1)	289.0 (0.1)	534.0 (0.2)	-	121.1 (.5)	-
0.02 M $TlNO_3$ ((4))	286.8 (0.2)	288.2 (0.2)	533.3 (0.2)	-	120.4 (0.2)	-
0.02 M TlDS ((4))	286.4 (0.2)	287.8 (0.4)	533.2 (0.4)	-	120.4 (0.4)	-
0.02 M $AgNO_3$ ((4))	287.0 (0.3)	288.5 (0.3)	533.4 (0.3)	-	-	368.8 (0.4)
0.02 M AgDS ((4))	286.5 (0.1)	288.2 (0.2)	533.2 (0.3)	-	-	369.0 (0.2)

[a] Six microliters of solution are used.
[b] Class II C 1s photoelectron.
[c] Class III C 1s photoelectron.
[d] Number in double parentheses, (( )), are the number of samples analyzed.
[e] Number in parentheses, ( ), represents the standard deviation.
[f] Values in brackets, [ ], are the I $3d_{5/2}$ binding energy as observed on the compound's pellet.

(ranging from 0.3 to 1.5 eV). The variability is within the error of the curve fitting process.

Elemental Ratios from XPS Analysis of Treated Papers

The absolute amount of an element present within the depth sampled by XPS is difficult to determine; however, the elemental ratios are readily obtained from the ratios of the normalized areas of the corresponding peaks. The normalized areas of O 1s, I $3d_{5/2}$, Tl ($4f_{5/2}$ and $4f_{7/2}$), and Ag $3d_{5/2}$ were compared to class II and III C 1s peaks obtained through a peak synthesis of C 1s peak. The results are given in Table 13. The addition of 6 μL of the oxygen containing organic molecules used in this study at concentrations of approximately 10^{-3} M do not alter significantly the oxygen to carbon ratio obtained from untreated filter paper under the experimental conditions used.

Iodide and Tl(I) ratios (I/C and Tl/C ratios in Table 13) from papers treated with 0.1 M KI and 0.1 M $TlNO_3$ are practically the same within the experimental error of the analysis, while the Ag/C ratio for papers treated with 0.02 M $AgNO_3$ is larger than those obtained for 0.5 M KI. Both I^- and Tl(I), with ionic radii of 2.12 and 1.54 Å, respectively (87), seemed to be equally retained on the surface as examined by XPS, despite their charge differences. However, silver with smaller ionic radius (1.26 Å) (87) is retained to a greater extent. This is interesting since this factor had not been considered before when comparing the effectiveness of different heavy atoms as enhancers of the RTP.

Table 13. Elemental Ratios From XPS Analysis of Treated Papers.

Spotting Solutions[b,c]	Elemental Ratios[a] O/C[d]	I/C[d]	Tl/C[d]	Ag/C[d]
Potassium Iodide				
0.1 M [1]	0.89	0.0023	-	-
0.5 M [1]	0.90	0.0073	-	-
1.0 M [3]	0.83 (.01)	0.013 (.002)	-	-
2.0 M [1]	0.84	0.032	-	-
Thallous Nitrate				
0.1 M [4]	0.82 (0.03)	-	0.0030 (0.0006)	-
0.02 M [4]	0.82 (0.10)	-	0.0016 (0.0004)	-
Thallium Dodecyl Sulfate				
0.02 M [4]	0.72 (0.04)	-	0.11 (0.02)	-
Silver Nitrate				
0.02 M [4]	0.88 (0.11)	-	-	0.0095 (0.0006)
Silver Dodecyl Sulfate				
0.02 M [4]	0.84 (0.09)	-	-	0.072 (0.018)
3,5-Diiodotyrosine				
0.002 M [3]	0.86 (0.05)	0.018 (0.002)	-	-
5-Hydroxytryptophan				
0.02 M [3]	0.87 (0.02)	-	-	-
Carbaryl				
0.002 M [1]	0.80	-	-	-

[a] The element's normalized area is ratioed to the added normalized areas of class II and III C 1s photoelectron peak.
[b] Six µL are used for spotting.
[c] Numbers in brackets are the number of samples analyzed.
[d] Numbers in parentheses represent the standard deviation.

There is a linear increase of the iodide ratios with increasing concentration of iodide in the solution as observed from the results in Table 13. The "heavy atom effect" of iodide on the RTP response of several phosphors is known to depend on the iodide concentration in solution (15,34). The increase in the phosphorescence intensity of 3-indolebutyric acid and of caffeine (see chapter 3), within the range of I^- concentrations of 0.1 M to 2.0 M correlates well with the results of the XPS studies: a linear increase in the phosphorescence intensity due to a proportional increase in the amount of I^- ions available at the surface of the paper. The iodine photoelectron peaks obtained from the surface of the papers treated with solutions of low iodide concentration (< 0.5 M) are very small, resulting in a small signal to noise ratio. There are not enough iodide ions at the surface of these samples which explains the need to use heavy atom solutions with a relatively high concentration (with respect to the analyte concentration) for the "heavy atom effect" to be observed.

Thallous salts are less soluble in water than iodide salts (100), and concentrations exceeding 0.1 M are difficult to prepare. Generally 0.1 M Tl solutions are used for RTP when Tl(I) is used as enhancer (17,34,101). The Tl ratios obtained from XPS analysis of paper spotted with 0.1 M Tl(I) are comparable to those obtained from papers spotted with 0.1 M I^-. From the heavy atom response curves, we know that this concentration of I^- is not enough to produce maximum RTP response. By analogy, solutions with higher concentration of Tl(I) should be used for optimum RTP response.

The iodine-carbon ratio for samples treated with 3,5-diiodotyrosine is a measure of the number of molecules present at the surface (see Table 13). Aqueous solutions of KI at a concentration of 1.0 M are commonly used in RTP studies. It is interesting to notice that although the 1.0 M KI solution is about three orders of magnitude more concentrated than 3,5-diiodotyrosine in solution (0.002 M), the relative number of I^- ions on the surface is the same within experimental error as the number of molecules of 3,5-diidotyrosine (refer to 0.014 I/C ratio for 1.0 M KI versus 0.018 for 0.002 M 3,5-diiodotyrosine). This seems to indicate a better retention of the organic molecule on the surface; the heavy atoms penetrate to a greater extent into the bulk of the filter paper.

Heavy Atom-Containing Surfactant Salts

Examination of the elemental ratios obtained for papers treated with 0.02 M solutions of $TlNO_3$, TlDS, $AgNO_3$, and AgDS (Table 13) reveals that both TlDS and AgDS are better retained on the surface of the paper than their corresponding salts. The ratios of silver and thallium increase by a factor of 7.6 and 69, respectively, when the surfactant salts of the ions are used in place of the nitrate salts. This correlates with the 7- and 9.5-fold increase in the RTP of carbaryl in the presence of AgDS and TlDS, respectively, compared to the intensities obtained when $AgNO_3$ and $TlNO_3$ are used as enhancers (see Appendix B). The use of a heavy atom-containing surfactant salt in place of the commonly used inorganic salt increases the intensity of the phosphorescence emission due probably to the increased availability of the heavy atom enhancer at the surface. The long

hydrocarbon chain seems to be retained better at the surface; this is evident from the change observed in the shape of the C 1s peak which appears to be slightly skewed (Fig. 12) in relation to the usual symmetrical carbon peak, indicating a greater contribution from class I carbon atoms. The fractional area corresponding to class I carbon atoms increased by a factor of 1.8 and 1.7 for AgDS and TlDS treated papers, respectively, when the surfactant salts were used in place of the nitrate salts. The better retention of the hydrocarbon chain results in improved retention of the heavy atom ion associated to the molecule.

Depth Profile

Grazing angle experiments were performed to examine surface layers of different thickness. The depth examined by XPS depends on the mean free path of the electrons in the cellulose polymer. There is some controversy regarding the mean free escape depth for organic polymers, λ; values ranging from 15 to 100 Å have been reported (93). Nevertheless, the number of layers examined by XPS for a given sample depends on the take-off angle θ (the angle between the electron path to the detector and the surface of the sample) (94) (see Fig. 11). The sample depth, D, is proportional to the man free path, λ, and the angle of electron exist, θ, ($D\alpha \lambda(\sin\theta)$. Since λ is constant for a given material and electron energy, decreasing θ results in smaller values of D.

Due to the rough and irregular surface of the filter paper, the data obtained are interpreted in a qualitative way (no correction is made for the roughness of the surface). Table 14 contains the results

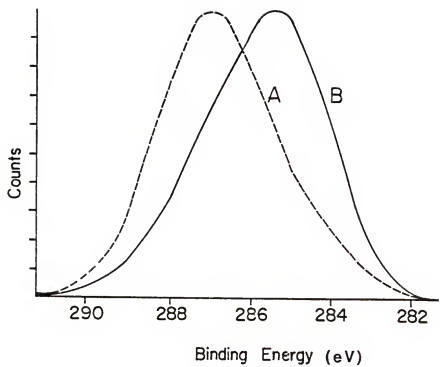


Fig. 12. Spectra of C 1s photoelectron peaks of untreated filter paper Whatman No. 1 (A) and of the filter paper after treatment with 6 μL of 0.02 M AgDS (B).

Table 14. Grazing Angle Experiments on Untreated Filter Paper.

Angle	Element	Binding Energy (eV)	Fractional Areas[a]	Ratio to ($C_{II} + C_{III}$)
90°	C_I	285.0	10	-
	C_{II}	286.4	77	-
	C_{III}	287.6	13	-
	O	532.8	-	0.75
45°	C_I	285.0	9	-
	C_{II}	286.7	78	-
	C_{III}	288.2	14	-
	O	533.0	-	0.76
10°	C_I	285.0	12	-
	C_{II}	286.9	69	-
	C_{III}	287.9	17	-
	O	533.0	-	0.74

[a] Fractional areas of the order of 5-20% can vary by a factor of ± 2; fractional areas greater than 50% can vary by less than ± 25%.

of the angle variation experiments for an untreated filter paper. No significant differences in the distribution of the different classes of carbon atoms or on the oxygen ratios within the sample depth examined is observed.

Similar experiments were done for filter papers treated with 1.0 M KI and with a DMF saturated solution of the platinum complex. A ratio of 0.016 and 0.019 was obtained for I/C and Pt/C, respectively at 90° grazing angle. When the angle was changed to approximately 10°, an I/C and Pt/C ratio of 0.015 and 0.014 was obtained, respectively. No significant changes are observed in these ratios as a function of grazing angle. The luminescent molecule and the heavy atom probe appear to be evenly distributed throughout the sampling depth examined by XPS.

Concluding Comments

Through XPS analysis of the surface of filter papers used as supports in RTP, it was possible to assess the presence of heavy atoms and of molecular probes on the surface and to determine the relative amounts present. The initial results obtained through this research on the extent of penetration of the molecules into the bulk of the filter paper and on the relative amounts of molecules compared to that of the heavy atoms on the surface needs to be confirmed through the use of different and better molecular probes. The requirements to be met by the compound to be used as a probe makes the selection difficult. The ideal probe for these types of studies has yet to be found.

With better resolution (e.g., the resolution obtained through the use of monochromatized X-rays) and larger surface coverages, it should be possible to observe chemical interactions between phosphor, heavy atom and/or substrate.

CHAPTER 5
CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH IN SOLID SUBSTRATE RTP

Room Temperature Phosphorescence as an Analytical Technique

Solid substrate RTP is a sensitive analytical technique suitable for the analysis of a variety of organic compounds, many of which are of interest in the biomedical area. The figures of merit (sensitivity, useful analytical range, and limits of detection) achieved in the analysis of the indole derivatives under optimum experimental conditions demonstrate the capabilities and applicability of the technique. Limits of detection in the nanogram region were obtained for all the compounds studied, including diazepam.

Diazepam is of special interest. This widely prescribed tranquilizer is one of the most encountered drugs in a toxicological laboratory. An assay for diazepam was developed based on its RTP properties on filter paper. The optimum experimental conditions, specific for diazepam, were determined. The selectivity of the analysis allowed for the determination of diazepam in the presence of other non- or weakly phosphorescent compounds. The successful application of solid substrate RTP to the analysis of diazepam in serum and in tablet formulation indicates that RTP has the potential of becoming a routine screening method of analysis for a variety of compounds. This is especially true for compounds of biomedical interest for which a simple, sensitive technique, capable of

automation (102), which will only require a small amount of sample, is desired.

In the application of RTP as an analytical technique, it is important to remember that the search for a good solid substrate, the use of a convenient heavy atom and a solvent system, and the consideration of the molecular structure of the analyte are critical factors for improving the selectivity and sensitivity of RTP.

Mechanisms of Interactions

The mechanisms of interactions which are responsible for RTP as observed from different solid substrates are varied and depend on the microenvironment that surrounds the phosphor. The most frequently postulated type of chemical interaction is hydrogen bonding. The interactions of the protonated molecule of diazepam with the different substrates and that of the indoles with the anionic paper DE-81 appears to involve hydrogen bonding.

Hydrogen bonding was not the only mechanism considered. Interactions between the heavy atom and the matrix must be taken into account and could play an important role in the observation of RTP. The possible interaction between the Hg(II) ions with the phosphate groups of the P-81 (diazepam study), or of Tl(I) with the carboxylate groups of the carboxymethylcellulose resin, CM 23 (indole study), explains the observed results under the particular experimental conditions. Both P-81 and CM 23 are cation exchangers, both are good substrates when positive heavy atoms are used as enhancers of RTP. The heavy atom plays an important role not only as enhancer of the phosphorescence but also in establishing the required rigidity for the

phosphor, through their interactions with both the support functional groups and the phosphorescent molecule.

The interactions that prevail in the "dry state" will depend on the type of support used (active sites at the surface), the structure of the phosphor (functional groups, electron density distribution, etc.), the presence of heavy atoms, and the wet chemistry occurring in solution before the spotting process and at the "wet surface" at the moment of spotting. The latter factor is crucial and the chemistry occurring is difficult to elucidate at this stage since the entire environment surrounding the phosphor at the wet surface must be considered. Neutralization reactions, hydrogen bonding, complex formation, and electrostatic interactions between heavy atom, support and/or phosphor may occur and be a decisive factor in the observation of RTP of the compound. For example, the neutralization of theophylline by DE-81 free hydroxyl groups during the wet stage does not allow for the observation of RTP under these conditions. Also, phosphorescence can be observed from the protonated methylxanthines when these are spotted on DE-81 or P-81, but not from Whatman No. 1, since both DE-81 and P-81 can neutralize the acidic effects of the solvent. Another example is the drastic decrease in the phosphorescence of the carboxylate salts of the indolecarboxylic acids on DE-81 due probably to the electrostatic repulsion between the free hydroxyl groups of DE-81 and the carboxylic groups of the salts of the indoles.

The research undertaken has provided a better understanding of the conditions necessary for the observation of RTP from the compounds

studied and of the possible mechanisms of interactions that prevail under different microenvironments. Additional research will answer basic questions on matrix packing, matrix isolation and rigidly-held mechanism.

Filter Paper as RTP Substrates

The ideal substrate for RTP has not yet been found. At present, filter papers are the most efficient and commonly used supports for RTP. Several disadvantages in the use of filter papers hinders the development of RTP as an analytical technique. The ubiquitous background phosphorescence determines the limits of detection in many instances. A method based on photolysis of the paper has been proposed (46) and can reduce significantly the background levels. A greater use of this method will help in reducing the spectral interferences and improving the limits of detection (LOD), resulting in an increased applicability of RTP.

There are other problems associated with the use of filter papers which must be considered. Penetration of the phosphor and of the heavy atom can be extensive. This affects both the sensitivity and LOD, since a significant amount of molecules and ions are lost within the bulk of the filter paper. The XPS studies of the surface of the paper showed that the extent of penetration of the heavy atoms or ions exceeds that of the organic compounds. Since a minimum amount of ions are required at the surface for the observation of the "heavy atom effect" this explains the need to use heavy atoms in concentrations several orders of magnitude greater than the phosphor concentrations,

as confirmed from the heavy atom response curves determined for I^- in the RTP of 3-indolebutyric acid and caffeine. It was interesting to observe that I^- and Tl(I) seemed to be equally retained at the surface of Whatman No. 1 but Ag(I) showed a different behavior. The amount of heavy atom retained at the surface of the support is expected to vary depending on the particular heavy atom and the type of support used. This should be considered when comparing "heavy atom effects" of different ions.

Care must be taken to ensure an adequate heavy atom coverage of the surface. If inorganic salts of the heavy atoms are used, their concentration in the spotting solution must be large enough to provide sufficient heavy atom ions at the surface. For example, in order to achieve optimum experimental conditions when inorganic thallous salts are used on filter paper, the solutions should be more concentrated than 0.1 M. Response curves of the heavy atoms for a particular phosphor on a given support will indicate the optimum concentration for the heavy atom.

Both migration and penetration of the ions and molecules deposited at the surface of filter papers could be minimized by improving the technique for sample deposition. This is an open area of research. Alternatively, the use of heavy atom-containing surfactants could provide an answer to this problem by insuring an adequate and more uniform surface coverage of heavy atoms. An increased availability of Tl(I) and of Ag(I) was observed with the use of TlDS and AgDS, as confirmed through XPS analysis of filter papers spotted with these salts and through the enhancement observed in the

RTP of carbaryl when surfactant salts are used in place of inorganic salts.

The use of surfactants may also have an effect on the retention of the luminescent molecule on the surface, on the rigidity achieved at the "dry stage," and in the phosphor luminescent properties in general. The association of the luminescent compound with the surfactant molecules can be very beneficial in the RTP process. The presence of a heavy atom-containing surfactant on the surface of a support, such as filter paper, together with the analyte could become a very successful combination for RTP. Additional research in this area is strongly recommended.

Since the ideal substrate has not yet been found and filter papers are very effective as substrate for RTP, research oriented toward minimizing the problems associated with their use is desired. In addition to the use of surfactants (with and without heavy atoms), penetration of the molecules might be reduced by improving the method for spotting the analyte and the heavy atoms on the surface. Research on the use of sprays or nebulizers capable of depositing very small droplets in the surface, in known amounts, is needed. Evaporation of the solvent from smaller droplets is faster and both migration and penetration of the molecules into the bulk of the paper would be greatly minimized.

Use of surface techniques (e.g., XPS) capable of monitoring the physical and chemical processes occurring at the surface is recommended. With adequate molecular probes, the extent of penetration of both heavy atoms and luminescence molecules can be

determined. X-ray photoelectron spectroscopy can be a very valuable tool in the search for new or for improved substrates/techniques for room temperature phosphorescence.

APPENDIX A
QUANTITATION OF X-RAY PHOTOELECTRON SPECTROSCOPY DATA

The intensity of the X-rays photoelectron peaks from solid samples depend on the intensity of the X-rays, the probability of X-ray/atomic orbital interaction, i.e., the photoionization cross-section, the probability that the photoelectron will actually be emitted into the vacuum, the probability that once emitted into the vacuum it is actually collected and detected by the spectrometer, and the concentration of the element present in the sample (93). The experimentally determined peak intensity (area), $I_{i,k}$, for the kth shell of atom i in the sample can be expressed (in simplified form) as:

$$I_{i,k} = \sigma_{i,k} \lambda_{i,k} N_i F T_{i,k}$$

where $\sigma_{i,k}$ is the photoionization cross-section for the kth shell of atom i; $\lambda_{i,k}$ is the mean free path for the kth electron of atom i in the sample of interest; N_i is the volume density of element i in the surface volume examined by XPS, F is the X-ray flux incident on the sample, and $T_{i,k}$ is the instrument transmission function at the kinetic energy of the electrons of the kth shell of atom i (93).

The mean free path, λ, is defined as the depth from which 1/e of the emitted electrons reach the surface; it is a function of the kinetic energy of the ejected electron and the solid matrix (93).

There is disagreement in the literature regarding the experimental values for mean free paths and in the theoretical development of such values. Wagner et al. (103) have shown that most of the available experimental data can be interpreted as the power function:

$$\lambda_{i,k} = C\, E_{i,k}^{m}$$

where C and m are functions of the solid matrix and $E_{i,k}$ is the kinetic energy of kth shell electron from atom i. They have shown that for kinetic energies greater than 300 eV and for organic solids, m is in the range of 0.7 to 1.0.

$T_{i,k}$ and $\sigma_{i,k}$ are also functions of the kinetic energy of the electrons and thus of the binding energies. The photoionization cross-section, $\sigma_{i,k}$, is a measure of the efficiency with which the X-rays are absorbed by the atoms in the sample (94). It is the most important factor in doing semi-quantitative work with XPS. If $\sigma_{i,k}$ is large for a particular atomic subshell from which an electron is being observed, then the sensitivity for this element will be high. The sensitivity factors calculated and tabulated by Scofield (104) were used in this research.

In principle, the intensity or area of the photoelectron peak is directly related to the number of atoms per unit volume in the sample. In practice, the exact value of the parameters may be difficult to obtain; therefore, the absolute number densities are seldom reported. However, the relative numbers of different elements in a sample may be deduced from the relative intensities of their

respective photoelectron peaks. By forming the ratio the common terms X-ray intensity and matrix parameters cancel out. For a homogeneous sample, the relative intensity for photoelectron peaks from two different atoms, 1 and 2, can be calculated (98) from the expression:

$$\frac{I_1}{I_2} = \frac{\sigma_{i,1}\ \lambda_{i,1}\ T_{i,1}\ N_1}{\sigma_{j,2}\ \lambda_{j,2}\ T_{j,2}\ N_2}$$

The mean free paths, $\lambda_{i,k}$, may be estimated from the kinetic energies of the emitted electrons. The transmission function, $T_{i,k}$, depends not only on the kinetic energy of the electrons ($E_{i,k}$) but also on the particular spectrometer design. For the Kratos XSAM 800, the mean free path factor is combined with the transmission function. The following equation is used in the normalization process:

$$\lambda_{i,k}\ T_{i,k} \approx (E_{i,k})^{1.75}$$

The relative number of atoms 1 to atoms 2 is obtained from the ratio of their normalized areas, A_N. The background subtracted areas, A_{BS}, of the photoelectron peaks are normalized with respect to their photoionization cross-section and their kinetic energy to the 1.75 power, and with respect to the dwell time and the number of sweeps used in the collection of data for the particular photoelectron peak:

$$A_{N,i,1} = \frac{A_{BS,i,1}}{(\sigma_{i,1})(E_{i,1})^{1.75}\ (\text{Dwell Time})(\text{Scans})_{i,1}}$$

The ratio of the number of atoms 1 to atoms 2 is

$$\frac{N_1}{N_2} = \frac{A_{N,i,1}}{A_{N,j,2}}$$

APPENDIX B
EFFECTS OF HEAVY ATOM CONTAINING SURFACTANTS IN THE ROOM TEMPERATURE PHOSPHORESCENCE OF CARBARYL

Introduction

The ideal substrate for RTP should provide a rigid matrix for the phosphor, protect it from potential quenchers, and allow for most of the luminescent and heavy atom compound to remain at the surface. In this research, the surface of filter paper was modified by the use of surface active agents. Surfactants are amphipathic molecules which can be anionic, cationic or nonpolar in nature (105). They have a long nonpolar hydrocarbon end and a polar end that is expected to interact strongly with the hydroxyl groups of the cellulose polymer.

Although micelles, which are aggregates of surfactant molecules, have been used with much success to observe RTP in solution (106,107), there is no report in the literature on the use of surfactants for solid substrate RTP. Recently, Alak et al. (108) published their results on the effect of surfactant spray reagents on the fluorescence of polycyclic aromatic hydrocarbons and (dansylated) amino acids. They observed luminescent enhancements with the use of the surfactant sprays when silica and alumina TLC plates were used, but no effect or a modest decrease in luminescence when reverse phase, cellulose, or polyamide plates were used.

The effects of the presence of heavy atom-containing surfactant salts on the surface of papers used as support for RTP was observed.

Salts of thallium(I) dodecyl sulfate (TlDS), and silver (I) dodecyl sulfate (AgDS) were prepared, characterized, and used as heavy atom enhancers of the phosphorescence of carbaryl. The RTP properties of carbaryl spotted on filter paper Whatman No. 1 and in the presence of thallium or silver surfactant salts are compared with those observed when the nitrate salts of thallium and silver are used. Solutions 0.005 and 0.02 M in the thallous and silver salts were prepared. The effects of KI, NaDS, and cetyltrimethylammonium bromide (CTAB) on the phosphorescence of carbaryl were also observed.

Experimental

Apparatus

Phosphorescence spectra were obtained using a Perkin-Elmer LS-5 spectrophotometer. A delay time (after the light pulse) of 0.1 ms and an observation time (gate time) of 9.0 ms were used for the measurement of the phosphorescence of carbaryl.

Reagents

The reagents KI, $AgNO_3$, $TlNO_3$, NaDS, and CTAB were used without purification. Nanopure deionized water, absolute ethanol, and isopropanol were used as solvents. Table 1 contains a list of the suppliers for all the reagents used in the study. Whatman No. 1 was used as support.

Procedures

The preparation and characterization of the heavy atom-containing surfactant salts is described in chapter 4.

A 3 μL aliquot of the carbaryl ethanolic solution (20 μg/mL) was spotted onto a 1 cm diameter disk of filter paper Whatman No. 1. The sample was dried under a stream of nitrogen for 20 min and the RTP measurements were then performed. Subsequently 3 μL of the heavy atom enhancer solution were added to the same spot and the drying and measurement procedure repeated. The results obtained before and after the addition of the different heavy atom ion solutions are compared.

Results and Discussion

Spectral Characteristics

The excitation or emission spectrum of carbaryl does not change in the presence of the thallous salts ($TlNO_3$ and TlDS) or the surfactant salts NaDS and CTAB. The emission spectrum shows two maxima at 483 and 522 nm with a shoulder at about 557 nm. The excitation spectrum shows a peak at 234 nm, a series of smaller, unresolved peaks at 256, 268, and 282 nm, and a shoulder at about 291 nm. Figure 13 shows the RTP spectrum of carbaryl in the presence of TlDS (0.005 M). When silver salts of low concentration (0.005 M) are used, the emission peak at 488 nm shows a significant decrease in intensity. When the concentration of the silver salt is increased to 0.02 M, the peak at 488 nm disappears almost completely and a shoulder at 490 nm appears in its place. A broad emission band with a maximum at 516 nm is then observed (Fig. 14).

RTP Intensities

The effects of the different Tl(I) and Ag(I) salts, KI, NaDS and CTAB on the phosphorescence intensity of carbaryl are listed in Table 15. The enhancement factors vary from 1.6 to 20 depending on the type

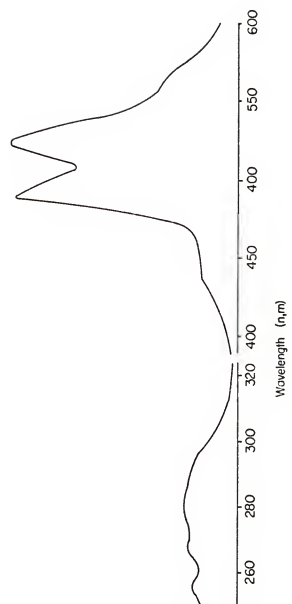


Fig. 13. Room temperature phosphorescence spectrum of 3 µL of carbaryl (20 µg/mL) in ethanol) spotted on Whatman No. 1 and in the presence of 3 µL of TIDS (0.005 M).

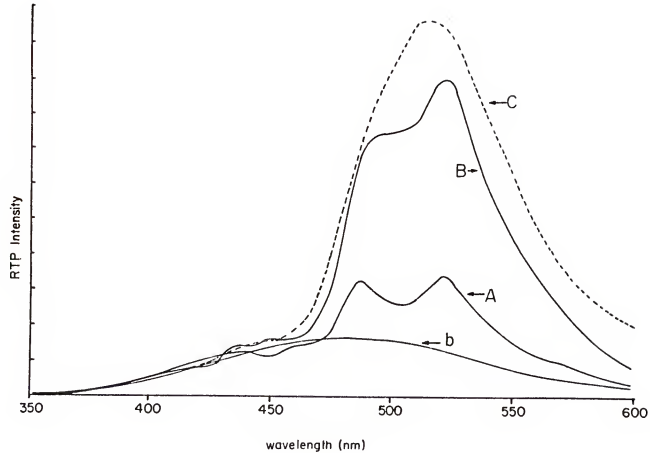


Fig. 14. Effect of silver salts on the RTP emission spectrum of carbaryl (3 µL of 20 µg/mL in ethanol) on Whatman No. 1; b is the background emission; A, B, and C are the emission spectra of carbaryl with no (A), 0.005 M (B), and 0.02 M (C) of AgDS present. Ordinate values for the different spectra do not correspond. (See Table 15 for relative intensities.)

Table 15. Effect of Heavy Atom Inorganic and Surfactant Salts on the RTP Emission of Carbaryl.

Compound (Concentration, M)[a]	I_c/I_o[b]
KI (0.005)	3.4 ± 1.2
NaDS (0.005)	1.6 ± 0.1
$AgNO_3$ (0.005)	2.3 ± 1.3[c]
AgDS (0.005)	6.3 ± 2.5[c]
$AgNO_3$ (0.02)	2.7 ± 0.4[c]
AgDS (0.02)[d]	19.0 ± 5.4[c]
$TlNO_3$ (0.005)	1.8 ± 0.6
TlDS (0.005)	8.6 ± 2.7
$TlNO_3$ (0.02)	2.1 ± 0.1
TlDS (0.02)[e]	20.0 ± 5.9
CTAB (0.02)	3.8 ± 0.9

[a] Heavy atom compound solution in water. Three microliters of 20 µg/mL of carbaryl (in ethanol) are used.
[b] Ratio of the net signal in the presence of the heavy atom compound (I_c) to the net signal in its absence (I_o).
[c] Partial or total destruction of the fine structure in the emission spectrum occurs.
[d] Solvent used is isopropanol.
[e] Solution in ethanol.

and amount of enhancer used. Although the NaDS molecule does not contain a heavy atom, a 1.6 enhancement factor is observed. The presence of NaDS on the surface of the paper could conceivably provide other sites of interaction for the phosphor resulting in increased rigidity of the molecule and, therefore, a higher intensity of the phosphorescence emission.

The enhancement factors of the least concentrated solutions (0.005 M) of TlDS and AgDS are higher than those obtained from solutions of KI, $AgNO_3$ or $TlNO_3$ of similar concentration. A comparison of the 0.005 M AgDS and TlDS with their corresponding nitrate salts shows increased intensity in the net phosphorescence signal by factors of 2.7 and 4.8, respectively.

Increasing the concentration of the surfactant salts in the spotting solution has a marked effect on the intensity of the phosphorescence. When the concentrations of AgDS and TlDS is increased from 0.005 to 0.02 M, a 2- to 3-fold increase in the intensity of carbaryl emission is observed. A similar increase in the concentration of the nitrate salts of Ag(I) and Tl(I) only produces a 1.2-fold increase in the intensity. The net phosphorescence signal in the presence of the surfactant salts (spotted from 0.02 M solutions) is higher than that in the presence of the corresponding nitrate salts by a factor of 7.0 and 9.5 for silver and thallium, respectively. Since the heavy atom solution is spotted on the surface after the paper has been spotted with the phosphor and the initial measurements done, higher RTP enhancement factors are expected if AgDS or TlDS solutions are spotted on the paper prior to the phosphor, or if a

premixed solution of the phosphor with AgDS or TlDS is used. It is interesting to note that CTAB (which has a bromine atom in the molecule) when used at the same concentration as AgDS and TlDS, 0.02 M, is not as efficient a source of heavy atoms for carbaryl as are AgDS and TlDS. Bromine may not be an effective enhancer of the phosphorescence of carbaryl.

The uncertainty observed in the ratios presented in Table 15 are attributed directly to typical variations of the phosphorescence background signal of the paper and to the standard deviation present in the carbaryl signal which decays with sample exposure time to the exciting radiation. Although the measurements were performed after stabilization of the signal (1-2 min), significant reading to reading variations are still observed (see Table 15).

The increase in the phosphorescence intensity of carbaryl when surfactant salts are used is probably due to a combination of factors: increased availability of heavy atom at the surface (see chapter 4) and increased rigidity of the matrix due to the presence of the surfactant.

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BIOGRAPHICAL SKETCH

The author was born October 24, 1948, in San Juan, Puerto Rico. Her parents are Manuel Andino and Carmen P. Andino. She was raised by her grandmother, Dolores Barens, in Catano, P.R. In May 1966, she graduated from Santa Rosa High School in Bayamon, P.R., and in June 1966 began her undergraduate studies in chemistry at the University of Puerto Rico. Marta graduated magna cum laude in May 1970, and in August 1971 started graduate studies in inorganic-physical chemistry at the University of Puerto Rico. She obtained an M.S. degree in June 1974.

During the two years following graduation from the University of Puerto Rico, the author worked as a chemist for the Department of Natural Resources, State of Wisconsin, at Madison, Wisconsin. Her two children, Gerardo Jose and Maria Cristina, were born on July 10, 1975, and March 23, 1977, respectively. Marta worked as an instructor in the Interamerican University of Puerto Rico from August 1980 to May 1982. In the fall of that year she started graduate studies in analytical chemistry at the University of Florida, Gainesville, Florida, and has worked since that time toward the Ph.D. degree. She is a member of the American Chemical Society, the Society for Applied Spectroscopy, the College of Chemists of Puerto Rico, and Iota Sigma Pi.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James D. Winefordner, Chairman
Graduate Research Professor of
Chemistry

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William Weltner, Jr.
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gerhard M. Schmid
Associate Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Anna Brajter-Toth
Assistant Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Joseph J. Delfino
Professor of Environmental Engineering Sciences

This dissertation was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1986

Dean, Graduate School